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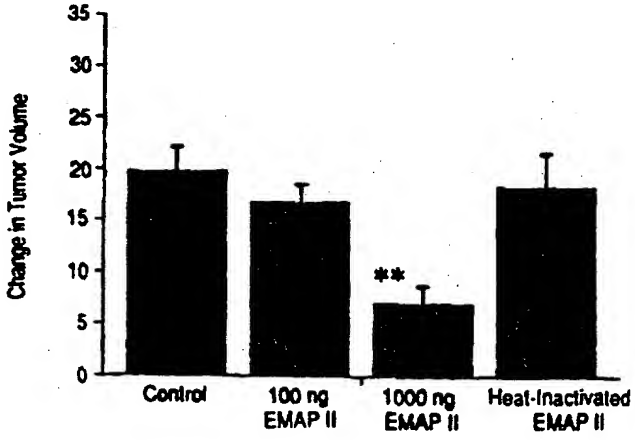
(57) Abstract

Tumors are treated by subcutaneously, intraperitoneally or intravenously administering endothelial monocyte activating polypeptide II (EMAP II) or an EMAP II-derived polypeptide. In addition, conditions involving the presence of excess blood vessels, for example retinopathy, are treated with EMAP II or an EMAP II-derived polypeptide.



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ANTIANGIOGENIC PROPERTIES OF ENDOTHELIAL-MONOCYTE ACTIVATING POLYPEPTIDE II

This application claims the benefit of U.S. Provisional No. 60/003,898, filed September 18, 1995, the contents of which are hereby incorporated by reference into the present application.

The invention disclosed herein was made with Government support under PHS Grant Nos. HL42833, HL42507, PERC, from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various references are referred to within parenthesis. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the sequence listing and the claims, or in the body of the text.

Background of the Invention

Recent studies have focussed attention on tumor neovasculature as a critical regulator of the growth of both primary and metastatic neoplastic lesion (Fidler I., 1994; Folkman J., 1989; Folkman J., 1995). Earlier studies emphasized the role of angiogenic factors, such as vascular endothelial growth factor (VEGF) (Plate K., 1992; Berkman, R.; Warren R., 1995; Kim J., 1993), acidic fibroblast growth factor (Maciag T., 1984), basic fibroblast growth factor (bFGF) (Shing Y., 1984), and angiogenin (Fett J., 1985; King T., 1991; Olson K., 1994), in promoting tumor growth and establishment of metastases. For example, in a transgenic murine model, a switch in phenotype from pancreatic adenoma to malignancy was closely tied to expression of angiogenic mediators (Kandel J., 1991), and blocking antibody to VEGF inhibited growth of explanted human tumors in athymic mice (Warren R., 1995; Kim J., 1993). Similar inhibition of experimental tumor growth has also been observed with

antibodies to angiogenin (Olson K., 1994) and bFGF (Hori A., 1991). Alternatively, recent work has delineated endogenous peptides with antiangiogenic activities, including angiostatin (O'Reilly M., 1994), thrombospondin (Dameron K.,1994) and glioma-derived angiogenesis inhibitory factor (Van Meir., 1994). Their presence appears to negatively impact on tumor growth either at the primary tumor site (thrombospondin) or at a site of distant metastases (angiostatin). Formation of the tumor vascular bed, as well as blood vessel formation in other situations, such as ischemia and atherosclerotic plaques (Shweiki, D., 1992; Knighton D., 1983; Kuwabara K., 1995; Sharma H., 1992; Chia M. 1991; Brogi E., 1993), is presumably controlled by the interaction of such positive and negative stimuli on endothelium in diverse vascular beds.

Endothelial-Monocyte Activating Polypeptide II (EMAP II) is a ≈20kDa protein isolated from Meth A fibrosarcoma cells (Kao J., 1992; Kao J., 1994), whose tumors exhibit characteristic vascular insufficiency manifested by heterogeneous pattern of thrombohemorrhage and central necrosis (Old L., 1986; Old L., 1961). EMAP II has been described in PCT International Publication No. WO 95/09180, published April 6, 1995, the contents of which are hereby incorporated by reference. These studies show that EMAP II has anti-angiogenic properties and results in suppression of tumor growth, likely due to perivascular apoptotic tissue injury and targeting of EMAP II to proliferating endothelial cells. These results demonstrate that endogenous or exogenously administered EMAP II controls blood vessel formation in a range of pathophysiologically relevant situations.

International Publication No. WO 95/09180 discloses that EMAP II administered in one intratumoral dose followed by one intravenous dose reduces the size of a tumor. WO 95/09180 also discloses that EMAP II has inflammatory activity. On the basis of its inflammatory activity one

would have expected that EMAP II would be toxic and therefore inappropriate for multiple administrations over a long period of time. Surprisingly, it has been found that multiple administrations of EMAP II decrease tumor size even without an intratumoral dose and without observed toxic effect. The ability to administer a therapeutically effective regimen of EMAP II without an intratumoral injection makes it possible to treat tumors whose small size makes it difficult or impossible to administer an intratumoral injection.

Retinal neovascularization is a major cause of blindness in the United States. Pathologic retinal angiogenesis is a common pathway leading to vision loss in disease processes such as retinopathy of prematurity, diabetic retinopathy, sickle cell retinopathy, and age related macular degeneration. Factors associated with retinopathy vascularization include hypoxia (cause of retinopathy of prematurity), diabetes, and known angiogenic factors such as Vascular endothelial growth factor (VEGF). The use of an established model of hypoxic induced retinopathy (Pierce, E. Jan. 1995; Smith, L., Jan. 1994) demonstrates that EMAP II, a protein associated with tumor antiangiogenesis, inhibits the neovascularization associated with retinopathy.

Summary of the Invention

This invention provides a method of treating a tumor in a subject, comprising administering to the subject an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to treat the tumor, wherein the endothelial monocyte activating polypeptide II is administered subcutaneously, intraperitoneally, or intravenously.

This invention provides a method of inhibiting the growth of endothelial cells, comprising contacting the endothelial cells with an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to inhibit growth of the endothelial cells.

Description of the Figures

Figure 1. SDS-PAGE of recombinant EMAP II. E. coli homogenate and pools of fractions containing EMAP II (See Fig. 2, below) were subjected to reduced SDS-PAGE (10-20% Tricine gels; 1-2 μg/lane) and protein visualized by silver staining. Lane 1, E. coli cell homogenate after centrifugation (12,000xg); lane 2, polyethylene imine supernatant; lane 3, Heparin Sepharose pool; lane 4, SP Sepharose pool; lane 5, Phenyltoyopearl pool; and lane 6, EMAP II formulated into PBS.

Figures 2A, 2B and 2C. Chromatographic steps in the purification of recombinant EMAP II. Fig. 2A. Heparin Sepharose. The polyethylene imine supernatant was applied to Heparin Sepharose in Tris buffer, washed and eluted with an ascending salt gradient. Fractions were monitored for absorbance at 280 nm and analyzed on SDS-PAGE and/or immunoblotting to identify the EMAP II pool (designated by the arrow and labeled EMAP II). (Fig. 2B) SP Sepharose. The Heparin Sepharose pool from (Fig. 2A) was concentrated, desalted and applied to SP Sepharose High Performance in MOPS buffer. After washing, EMAP II was eluted by an ascending salt gradient and pooled as above. Phenyl toyopearl. The SP Sepharose pool was adjusted to 2 M $(NH_4)_2SO_4$ and applied to Phenyl toyopearl in phosphate buffer with salt, washed, and EMAP II eluted with a descending salt gradient. The salt gradients are shown as (---), 0-1 M in NaCl (A-B), and (---)0-2 M $(NH_4)_2SO_4$ (C). Absorption at 280nm is shown by the solid line in each figure.

Figures 3A, 3B, 3C , 3D and 3E. Matrigel angiogenesis model: effect of EMAP II on bFGF-induced neovascularization. Mice received subcutaneous Matrigel implants and were sacrificed after 14 days to analyze new vessel formation by histologic examination and hemoglobin assay. Fig. 3A and 3C implant containing bFGF (100 μg/ml)/heparin (40 U/ml) shown at low and high power, respectively; Fig. 3B and 3D, implant containing bFGF/heparin + EMAP II (100 ng/ml) shown at low

and high power, respectively; Fig. 3E, results of hemoglobin (reported as percent of control, Matrigel with vehicle, arbitrarily defined as 100%). Results were evaluated by student t-test and $p<0.001$ comparing hemoglobin levels in the presence of EMAP II with control and bFGF. Magnification in Figures 3A-3C, 10x.

Figures 4A, 4B and 4C. Disappearance of ^{125}I-EMAP II from mouse plasma after IV or IP injection (Fig. 4A), precipitability of the tracer in trichloroacetic acid (Fig. 4B), and tissue accumulation (Fig. 4C). Fig. 4A. Mice received ^{125}I-EMAP II (0.26 μg) by either IV or IP injection and plasma was sampled at the indicated time points. The methods for data fitting and parameters of clearance are described in the text. Fig. 4B. Trichloroacetic acid precipitability of ^{125}I-EMAP II in spleen and B16 tumor harvested 12 hrs after IP injection as above. Tissue was homogenized, weighed, counted, and subjected to precipitation in trichloroacetic acid (20%). The mean ±SD is shown (n=12), and data were analyzed by student t-test; $p<0.001$. Fig. 4C. Deposition ^{125}I-EMAP II in tumor, spleen, brain and liver. Animals received IP ^{125}I-EMAP II, as above at time 0, and then 1 hr prior to harvest ^{51}Cr-labeled microspheres were infused IV. At the 12 hr point, animals were sacrificed, the indicated organs were removed, dried, weighed and counted. The mean ±SE is shown (n=4) and data analyzed by Mann-Whitney showed a $p<0.02$ comparing tissue counts in the tumor to spleen and brain.

Figures 5A, 5B, 5C, 5D, 5E, 5F and 5G. Effect of EMAP II on Lewis Lung Carcinoma (LLC). Mice were injected subcutaneously on day 1 with LLC cells, and then on days 3-15 received every 12 hrs IP either: vehicle alone (control), EMAP II (100 or 1000 ng) or heat-inactivated EMAP II (1000 ng). Fig. 5A. Change in tumor volume in each of the four groups (mean ± SD) is shown (n=40), and data analysis was performed as described in the text ($p<0.034$, Kruskal-Wallis; and $p<0.003$ Mann-Whitney). Figs. 5B-5E. Histology of LLC

tumors harvested from the indicated above groups on day 15: Figure 5B and 5C, vehicle alone, high and low power, respectively; Figure 50C and 5E, EMAP II (100 ng) high and low power, respectively; Figs. 5F-G. DNA fragmentation by in situ nick translation: Fig. 5F, vehicle alone and Fig. 5G, EMAP II (1000 ng).

Figures 6A, 6B, 6C, 6D, 6E and 6F. Effect of EMAP II on cultured endothelial cells (ECs). Figures 6A-6D. Effect on EC monolayer wound repair in vitro. A postconfluent monolayer of ECs was wounded (wound margin at upper right), and then either vehicle (Figures 6A, 6C) or rEMAP II (10 ng/ml; Figures 16B, 16D) was added. After 24 hrs of incubation, cultures were stained with rhodamine phalloidin (Figures 6A-6B) to display the actin-based cytoskeleton or with DAP-1 (Figures 6C-6D) to demonstrate the presence of apoptotic bodies, noted by arrows in Figure 6D. Figures 6E-6F. DNA fragmentation by ELISA of subconfluent ECs in normoxia or hypoxia (pO_2 ≈14 torr), exposed to rEMAP II as indicated. Data shown represent mean and, in each case, S.E. was less than 10%. These experiments were repeated a minimum of three times. Magnification: Figures 6A-6D, 128X.

Figures 7. PCR analysis of EMAP II transcripts in normal murine tissue. RNA was harvested from normal murine tissues as indicated, and processed for PCR as described in the text. The bands corresponding to the amplicons for EMAP II (400 bp) and ß-actin (560 bp) are shown by the arrows. A 100 bp ladder was used as the standard in the far left lane.

Figures 8A, 8B, 8C, 8D and 8E. Lung metastasis model with LLC. Mice received LLC cells subcutaneously and were observed until tumors reached a volume of ≥1.5 cm^3, at which time animals were treated with rEMAP II (1000 ng; IP every 12 hrs; N=8) or vehicle alone (control; N=6) for 72 hrs. Tumors were subsequently resected (there were no local recurrences), and the same treatment regimen was continued for the duration of the study, an additional 15 days. India

ink was instilled intratracheally to enhance visualization of metastases (pale areas) compared with normal tissue (dark areas). Gross appearance of lungs demonstrated many surface macrometastases in controls (Figure 8A) versus their marked suppression in rEMAP II-treated mice (Figure 8B). Histologic examination confirmed this impression (Figure 8C, vehicle-treated, and Figure 8D, EMAP II-treated; arrow in Figure 8D and Figure 8D inset indicate the presence of micrometastasis). In Figure 8E, surface lung metastases/nodules data from all animals was analyzed using Mann-Whitney ($p<0.009$); total surface metastases are shown in the Figure 8E (mean ± S.E.) and surface macrometastases (>2mm; mean ± S.E. for control and EMAP II-treated groups were 80±12.5% and 20±13%, respectively), counted using a calibrated ocular, are shown in Figure 8E (by student t-test $p<0.002$). These experiments were repeated four times. Marker bar, 1 cm (Figures 8A-8B); magnification Figure 8C-8D, 12.8X; and Figure 8D inset, 32X.

Figures 9A, 9B, 9C, 9D, 9E and 9F. Effect of rEMAP II on C6 gliomas implanted intracranially into rats and subcutaneously into mice. Figures 9A-9D. Intracranial C6 gliomas in rats. Figure 9A. C6 glioma cells were implanted stereotactically as described, and rats were maintained for 10 days, at which time they were divided into eight treatment groups as indicated. Tumor volume was evaluated on day 26 (after 16 days of treatment). ** and * indicate $p<0.0001$ and $p<0.005$, respectively, by Kruskal-Wallis. In Fig. 9A, the mean ±SE is shown. Figure 9B-9C. Intracranial tumors, derived from C6 glioma cells, were harvested from animals treated with vehicle (Fig. 9B and 9D; IT/IP) alone or EMAP II (Fig. 9C and 9E; IT/IP). Sections were stained with hematoxylin and eosin (9B, 9C)) or subjected to the TUNEL procedure (9D, 9E). Figure 9F. Subcutaneous C6 gliomas in nude mice. Tumor cells were implanted, animals were maintained for 3 days, and treatment with EMAP II was initiated for the next 24 days as described. At the end of the experiment, tumor volume was measured and data shown

represent the mean ± SE. The intracranial tumor experiments were repeated three times and the subcutaneous tumor studies were repeated twice.

Figures 10A, 10B, 10C, 10D and 10E. Effect of rEMAP II on vascular ingrowth into Matrigel implants impregnated with VEGF. Matrigel mixtures containing VEGF (100 ng/ml) were administered subcutaneously and, simultaneously, animals (N = 10 per group) received rEMAP II (1 μg; IP every 12 hrs) in vehicle or vehicle alone for the next 14 days. Implants were evaluated by hematoxylin and eosin staining (Figures 10A & 10C, treated with rEMAP II; Figures 10B & 10D, treated with vehicle) and quantitation of hemoglobin content (Figures 10E; mean ± SE; * indicates $P<0.01$). The experiments were repeated three times.

Figures 11A, 11B, 11C, 11D and 11E. Interaction of rEMAP II with cultured endothelial and C6 glioma cells. Figure 11A-11B. Human umbilical vein endothelial cells or C6 glioma cells in Medium 199 containing fetal calf serum (10%) were exposed to rEMAP II (10nM;A) or medium alone (B) for 24 hrs at 37°C, samples were harvested and subjected to TUNEL analysis as described. Figure 11C. Quantitation of apoptotic endothelial nuclei as a ratio of labelled nuclei/cells counted in each of ten high power fields in the presence of the indicated concentration of rEMAPII. * denotes P<x. 11D. Quantitation of labelled nuclei as in (C) when C6 glimoa cells in Dulbecco's MEM were incubated with the indicated concentration of rEMAPII or medium alone. Figure 11E. Radioligand binding study with ^{125}I-rEMAP II. Human umbilical vein endothelial cell monolayers or C6 glioma cells were incubated with the indicated concentrations of ^{125}I-rEMAP II alone or in the presence of an 100-fold excess of unlabelled rEMAP II. Specific binding is plotted and the curve indicates the best-fit using nonlinear least squares analysis. Parameters of binding on endothelial cells were: Kd= 1.9 nM and B=compared with C6 glioma cells. Similar radioligand

binding experiments on C6 glioma cells showed no specific binding.

Detailed Description

This invention provides a method of treating a tumor in a subject, comprising administering to the subject an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to treat the tumor, wherein the endothelial monocyte activating polypeptide II is administered subcutaneously, intraperitoneally, or intravenously. Many types of tumors can be treated according to this method. In a preferred embodiment the tumor is a carcinoma.

The term "EMAP II" refers to Endothelial Monocyte Activating Polypeptide II. The term "rEMAP II" refers to recombinant Endothelial Monocyte Activating Polypeptide II. EMAP II may also include variants of naturally occurring EMAP II. Such variants can differ from naturally occurring EMAP II in amino acid sequence or in ways that do not involve sequence, or both. Variants in amino acid sequence are produced when one or more amino acids in naturally occurring EMAP II is substituted with a different natural amino acid, an amino acid derivative or non-native amino acid. Particularly preferred variants include naturally occurring EMAP II, or biologically active fragments of naturally occurring EMAP II, whose sequences differ from the wild type sequence by one or more conservative amino acid substitutions, which typically have minimal influence on the secondary structure and hydrophobic nature of the protein or peptide. Variants may also have sequences which differ by one or more non-conservative amino acid substitutions, deletions or insertions which do not abolish the EMAP II biological activity. Conservative substitutions (substituents) typically include the substitution of one amino acid for another with similar characteristics such as substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. The non-polar (hydrophobic)

amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

Other conservative substitutions can be taken from Table 1, and yet others are described by Dayhoff in the Atlas of Protein Sequence and Structure (1988).

Table 1: Conservative Amino Acid Replacements

For Amino Acid	Code	Replace with any of
Alanine	A	D-Ala, Gly, beta-ALa, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, Beta-Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Met, D-Met

Lysine	K	D-Lys,Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val, Norleu
Phenylalanine	F	D-Phe,Tyr, D-Thr,L-Dopa,His,D-His, Trp, D-Trp, Trans 3,4 or 5-phenylproline, cis 3,4 or 5 phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4-carboxylic acid, D- or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O) D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr,Phe, D-Phe, L-Dopa, His,D-His
Valine	V	D-Val, Leu,D-Leu,Ile,D-Ile, Met, D-Met

Other variants within the invention are those with modifications which increase peptide stability. Such variants may contain, for example, one or more non-peptide bonds (which replace the peptide bonds) in the peptide sequence. Also included are: variants that include residues other than naturally occurring L-amino acids, such as D-amino acids or non-naturally occurring or synthetic amino acids such as beta or gamma amino acids and cyclic variants. Incorporation of D- instead of L-amino acids into the polypeptide may increase its resistance to proteases. See, e.g., U.S. Patent 5,219,990.

The peptides of this invention may also be modified by various changes such as insertions, deletions and

substitutions, either conservative or nonconservative where such changes might provide for certain advantages in their use.

In other embodiments, variants with amino acid substitutions which are less conservative may also result in desired derivatives, e.g., by causing changes in charge, conformation and other biological properties. Such substitutions would include for example, substitution of hydrophilic residue for a hydrophobic residue, substitution of a cysteine or proline for another residue, substitution of a residue having a small side chain for a residue having a bulky side chain or substitution of a residue having a net positive charge for a residue having a net negative charge. When the result of a given substitution cannot be predicted with certainty, the derivatives may be readily assayed according to the methods disclosed herein to determine the presence or absence of the desired characteristics.

Variants within the scope of the invention include proteins and peptides with amino acid sequences having at least eighty percent homology with EMAP II. More preferably the sequence homology is at least ninety percent, or at least ninety-five percent.

Just as it is possible to replace substituents of the scaffold, it is also possible to substitute functional groups which decorate the scaffold with groups characterized by similar features. These substitutions will initially be conservative, i.e., the replacement group will have approximately the same size, shape, hydrophobicity and charge as the original group. Non-sequence modifications may include, for example, in vivo or in vitro chemical derivatization of portions of naturally occurring EMAP II, as well as changes in acetylation, methylation, phosphorylation, carboxylation or glycosylation.

In a further embodiment the protein is modified by chemical

modifications in which activity is preserved. For example, the proteins may be amidated, sulfated, singly or multiply halogenated, alkylated, carboxylated, or phosphorylated. The protein may also be singly or multiply acylated, such as with an acetyl group, with a farnesyl moiety, or with a fatty acid, which may be saturated, monounsaturated or polyunsaturated. The fatty acid may also be singly or multiply fluorinated. The invention also includes methionine analogs of the protein, for example the methionine sulfone and methionine sulfoxide analogs. The invention also includes salts of the proteins, such as ammonium salts, including alkyl or aryl ammonium salts, sulfate, hydrogen sulfate, phosphate, hydrogen phosphate, dihydrogen phosphate, thiosulfate, carbonate, bicarbonate, benzoate, sulfonate, thiosulfonate, mesylate, ethyl sulfonate and benzensulfonate salts.

Variants of EMAP II may also include peptidomimetics of EMAP II. Such compounds are well known to those of skill in the art and are produced through the substitution of certain R groups or amino acids in the protein with non-physiological, non-natural replacements. Such substitutions may increase the stability of such compound beyond that of the naturally occurring compound.

In an embodiment of this invention the subject is a mammal. Examples of suitable mammalian subjects include, but are not limited to, murine animals such as mice and rats, hamsters, rabbits, goats, pigs, sheep, cats, dogs, cows, monkeys and humans. In a specific embodiment the agent is administered intraperitoneally.

By means of well-known techniques such as titration and by taking into account the observed pharmacokinetic characteristics of the agent in the individual subject, one of skill in the art can determine an appropriate dosing regimen. See, for example, Benet, et al., "Clinical Pharmacokinetics" in ch. 1 (pp. 20-32) of Goodman and

Gilman's The Pharmacological Basis of Therapeutics, 8th edition, A.G. Gilman, et al. eds. (Pergamon, New York 1990). In an embodiment of this invention the agent is administered in at least twenty doses. In a specific embodiment the agent is administered in about twenty-four doses. In an embodiment the agent is administered over a period of at least ten days. In a specific embodiment, the agent is administered over a period of about twelve days. In an embodiment of this invention, the frequency of administration is at least about one dose every twelve hours. In an embodiment the effective amount is from about 2.4 micrograms to about 24 micrograms. In an embodiment the effective amount is from about 100 nanograms to 24 micrograms per dose. In a more specific embodiment the effective amount is from about 100 nanograms to about 1000 nanograms per dose.

In an embodiment of the method described herein, the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDASRLDLRIG (C/R)IVTAKKHPDADSLYVEEVDVGEAAPRTVVSGLVNHVPLEQMQNRMVVLLCNLK PAKMRGVLSQAMVMCASSPEKVEILAPPNGSVPGDRITFDAFPGEPDKELNPKKKIWE QIQPDLHTNAECVATYKGAPFEVKGKGVCRAQTMANSGIK (SEQ I.D. No. ___), wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues. In a preferred embodiment the homology is at least about ninety-five percent.

In another embodiment of the method described herein the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDVSRLDLRIG (C/R)IITARKHPDADSLYVEEVDVGEIAPRTVVSGLVNHVPLEQMQNRMVILLCNLK PAKMRGVLSQAMVMCASSPEKIEILAPPNGSVPGDRITFDAFPGEPDKELNPKKKIWE QIQPDLHTNDECVATYKGVPFEVKGKGVCRAQTMSNSGIK (SEQ I.D. No. ___), wherein the sequence is truncated by from zero to about

three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues. In a preferred embodiment the homology is at least about ninety-five percent.

In a preferred embodiment of the method described herein the agent is endothelial monocyte activating polypeptide II. In a more specific embodiment, the EMAP II is murine EMAP II or human EMAP II. In an embodiment the endothelial monocyte activating polypeptide II is recombinant endothelial monocyte activating polypeptide II.

An advantage of the above-described method compared to treatment protocols involving intratumor injection is that tumors that are too small for intratumoral injection can be treated before they grow to a larger size. Accordingly, in an embodiment of this invention the tumor is too small for intratumoral injection. For example, in a specific embodiment, the diameter of the tumor is less than or equal to about two millimeters.

This invention provides a method of inhibiting the growth of endothelial cells, comprising contacting the endothelial cells with an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to inhibit growth of the endothelial cells. In an embodiment, the endothelial cells are aortic endothelial cells, for example bovine aortic endothelial cells.

This invention provides a method of inhibiting the formation of blood vessels in a subject, comprising administering to the subject an effective amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, thereby inhibiting the formation of blood vessels in the subject.

In an embodiment of this invention the subject is a mammal. Examples of suitable mammalian subjects include, but are not limited to, murine animals such as mice and rats, hamsters, rabbits, goats, pigs, sheep, cats, dogs, cows, monkeys and humans.

The agent may be administered according to techniques well known to those of skill in the art, including but not limited to subcutaneously, intravascularly, intraperitoneally, topically, or intramuscularly.

In an embodiment the effective amount is from about 10 nanograms to about 24 micrograms. In a specific embodiment the effective amount is from about 100 nanograms to about 1 microgram.

In an embodiment of the method described herein, the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDASRLDLRIG (C/R)IVTAKKHPDADSLYVEEVDVGEAAPRTVVSGLVNHVPLEQMQNRMVVLLCNLK PAKMRGVLSQAMVMCASSPEKVEILAPPNGSVPGDRITFDAFPGEPDKELNPKKKIWE QIQPDLHTNAECVATYKGAPFEVKGKGVCRAQTMANSGIK (SEQ I.D. No. ___), wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues. In a preferred embodiment the homology is at least about ninety-five percent.

In another embodiment of the method described herein the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDVSRLDLRIG (C/R)IITARKHPDADSLYVEEVDVGEIAPRTVVSGLVNHVPLEQMQNRMVILLCNLK PAKMRGVLSQAMVMCASSPEKIEILAPPNGSVPGDRITFDAFPGEPDKELNPKKKIWE QIQPDLHTNDECVATYKGVPFEVKGKGVCRAQTMSNSGIK (SEQ I.D. No. ___), wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one

hundred thirty-six carboxy-terminal residues. In a preferred embodiment the homology is at least about ninety-five percent.

In a preferred embodiment of the method described herein the agent is endothelial monocyte activating polypeptide II. In a more specific embodiment, the EMAP II is murine EMAP II or human EMAP II. In an embodiment the endothelial monocyte activating polypeptide II is recombinant endothelial monocyte activating polypeptide II.

This invention provides a method of treating a condition involving the presence of excess blood vessels in a subject, comprising administering to the subject an effective amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, thereby treating the condition involving the presence of excess blood vessels.

In an embodiment, the condition involves the presence of excess blood vessels in the eye. One such condition is retinopathy. In specific embodiments of the method the retinopathy is diabetic retinopathy, sickle cell retinopathy, retinopathy of prematurity, or age related macular degeneration.

The present invention provides for a method of treating a tumor in a subject, comprising administering to the subject an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to treat the tumor, wherein the endothelial monocyte activating polypeptide II is administered subcutaneously or intraperitoneally; and intravenously, intracranially, or intramorally. The tumor may be a glioblastoma. The agent may be administered intratumorally by positive pressure microinfusion.

The present invention further provides for a method for evaluating the ability of an agent to inhibit growth of endothelial cells, which includes: (a) contacting the endothelial cells with an amount of the agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide; (b) determining the growth of the endothelial cells, and (c) comparing the amount of growth of the endothelial cells determined in step (b) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit growth of endothelial cells.

The present invention provides for a method for evaluating the ability of an agent to inhibit the formation of blood vessels in a cellular environment, which comprises: (a) contacting the cellular environment with an amount of the agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide; (b) determining whether or not blood vessels form in the cellular environment, and (c) comparing the amount of growth of blood vessels determined in step (b) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit formation of blood vessels is the cellular environment.

As used herein, a cellular environment includes but is not limited to a cell culture system, cells in vivo, cells in vitro, an organ culture, an animal model system. A cellular environment may include a cells growing in a subject, a tumor cell culture system, an endothelial cell culture system, an embryonic cell culture system, an angiogenic cell culture system. A cellular environment may be either in vitro or in vivo. A cellular environment may include a hybridoma cell culture system.

The present invention provides for a pharmaceutical composition which comprises an agent capable of inhibiting

blood vessel formation and a pharmaceutically acceptable carrier. The carrier may include but is not limited to a diluent, an aerosol, a topical carrier, an aquous solution, a nonaqueous solution or a solid carrier.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

ANTI-TUMOR TREATMENT

Example 1: Endothelial-Monocyte Activating Polypeptide II, A Novel Antiangiogenic Protein, Suppresses Tumor Growth and Induces Apoptosis of Growing Endothelial Cells.

Materials and Methods

Cell culture. Bovine aortic endothelial cells (ECs) were isolated from calf aortae, grown in culture and characterized, based on the presence of von Willebrand factor and thrombomodulin, as described previously (Nawroth P., 1988). Bovine vascular smooth muscle cells were prepared by additional scraping of the aortae following removal of the endothelium, and were characterized based on the presence of smooth muscle cell actin (Gown A., 1985). Lewis Lung carcinoma cells (LLC), obtained from American Type Culture Collection (ATCC), NIH 3T3 cells (ATCC), and B16 (F10) cells were all maintained in high glucose defined minimal essential medium (DMEM; Gibco) containing fetal bovine serum. Meth A tumor cells were provided by Dr. Lloyd Old (Center for Cancer Research, NY), and grown as described (Old L., 1987; Old L., 1961).

Preparation of recombinant murine EMAP II and EMAP II ELISA. Recombinant EMAP II was prepared from E. coli (host HMS174[DE3]) transformed with a plasmid containing the coding sequence for mature EMAP II, as described previously (Kao J., 1994). A procedure was developed for purification of recombinant EMAP II on a preparative scale. Frozen (-80°C) E. coli cell paste was mixed 1:10 (w/v) with Tris-HCl (20mM; pH 7.4) containing octyl-ß-glucoside (0.1%) and an homogeneous suspension formed by agitation with a TURRAX® for 20 min (speed 60) at 4°C. The suspension was then disrupted by three passes through a TURRAX® Microfluidizer (Mode 110F) at 4°C. Polyethylene imine at pH 7 was then added to the homogenate to a concentration of 0.25%. The homogenate was left for 30 min on ice to precipitate cell debris and DNA. Solids were removed from the homogenate by centrifugation (5000xg; 30 min), the polyethylene imine

supernatant was retained and filtered (0.2 μm), and applied (3 mls sample ml of gel) to Heparin sepharose CL-4B (Pharmacia, Piscataway NJ; 120 ml bed volume) equilibrated in Tris-HCl (20 mM; pH 7.4) containing octyl-ß-glucoside (0.1%). After washing the column with the same buffer (20 ml/min), a linear ascending NaCl gradient (0 to 0.5M) in the wash buffer was applied. Fractions were pooled on the basis of purity by silver stained SDS-PAGE and by immunoblotting with antibodies prepared to the N-terminus of mature EMAP II (Kao J., 1992). EMAP II biological activity was measured based on induction of tissue factor in cultural endothelial cell, as described (Kao J., 1992).

The Heparin Sepharose pool was concentrated using an Amicon Stirred Cell (Amicon) with an Amicon YM10 Diaflo Ultrafilter to less than 100 ml. The retentate was desalted into 3-(Morpholino)-propane-sulfonic acid (MOPS; 25 mM; pH 6.9) on a Sephadex G25 (Medium Grade, Pharmacia) size exclusion column (480 ml bed volume). The pool was then applied to an SP Sepharose High Performance (Pharmacia) cation exchange column (55ml bed volume) run at a flow rate of 10 ml/min. After washing the column with MOPS buffer, EMAP II-containing fractions were eluted by application of a 0 to 0.5 M ascending linear salt gradient in MOPS. The pool was identified, assayed for total protein an biological activity as above.

EMAP II-containing fractions from SP Sepharose chromatography were adjusted to 2 M in $(NH_4)_2SO_4$ with solid $(NH_4)_2SO_4$ and applied to a Phenyl Toyopearl 650 M (Tosohaas) column (90 ml bed volume) equilibrated in sodium phosphate (20 mM; pH 7) containing 1 M $(NH_4)_2SO_4$. After washing with the above buffer, a descending gradient of salt (2 to 0M) in sodium phosphate (20 mM) was applied. EMAP II-containing fractions were pool and characterized as above.

EMAP II from in the Phenyl Toyopearl column eluate was concentrated to 3-5 mg/ml, and formulated into phosphate-

buffered saline (PBS; pH 7.4) by buffer exchange on a Sephadex G25 column (as above). Lipopolysaccharide (LPS) was removed using filtration through a Posidyne filter (Pall Corp.), and LPS levels were estimated using the Endospecy chromogenic assay (limit of detection <10pg/ml). Purified EMAP II, as well as EMAP II in fractions obtained during the purification procedure was subjected to N-terminal sequence analysis, mass spectrometry and SDS-PAGE. Samples were mixed with 1:0.5 (v:v) of Tricine SDS sample buffer with the addition of 1/10 volume of 1 M dithiothreitol, boiled for 5 min, and applied (1 μg/lane) to 10-20% Tricine gels (Novex) and electrophoresed in SDS Tricine running buffer at constant voltage (100 V) for about 2 hrs at room temperature. Protein was visualized by silver staining, and molecular weight markers (Novex) were run simultaneously. Immunoblotting was performed following SDS-PAGE by transferring protein to nitrocellulose in Tris-HCl (12 mM), glycine (96 mM; final pH 8.3) containing methanol (20%) using the Novex Western Transfer Apparatus at constant voltage (30 V) for 2-4 hr (4°C). Prestained, low molecular weight markers (Bio-Rad) were used to follow the transfer. Immunoreactive protein was visualized using rabbit anti-mature EMAP II N-terminal peptide IgG (0.1 μg/ml) followed by the Amplified Alkaline Phosphatase Goat Anti-Rabbit Immuno-Blot Assay Kit (Bio-Rad).

Antibody to EMAP II was prepared by standard methods (30), and was found to be monospecfic based on immunoblotting of plasma and cell extracts. This antibody was used to develop an ELISA to detect EMAP II antigen; cells or tissues were homogenized in the presence of protese inhibitors (phenylmethylsulfonyl fluoride, 1 mM; trasylol, 0.1%), centrifuged to remove debris, and the supernatant was diluted in carbonate/bicarbonate buffer (pH 9.6) and incubated in Maxisorb microtiter plates (Nunc) overnight at 4°C. Wells were washed with phosphate-buffered saline, excess sites were blocked with bovine serum albumin (1%) in phosphate-buffered saline for 30 min at room temperature,

and then incubated for 1 hr at 37°C with monospecific polyconal rabbit immune IgG against EMAP II dissolved in phosphate-buffered saline containing bovine serum albumin (1%). Primary antibody was revealed with peroxidase conjugated secondary antibody and o-phenylenediamine dihydrchloride as the chromogenic substrate. Concentrations of EMAP II were determined by comparison with a standard curve made with known amounts of recombinant murine EMAP II.

Matrigel model. Matrigel (Kleinman H., 1986; Passaniti A., 1992) (Collaborative Research) containing either EMAP II (100 ng/ml), bFGF (100 ng/ml) (Collaborative Research) and heparin (40 U/ml; Sigma), EMAP II (100 ng) + bFGF/heparin, vehicle alone (1% BSA), or heat-inactivated EMAP II (alone or with bFGF/heparin) were mixed at 4°C. Matrigel mixtures were injected subcutaneously into C57BL6/J mice (0.25 ml/site) at two sites per animal. The angiogenic response was analyzed at 7 and 14 days post-inoculation by routine histology and hemoglobin assay (Sigma).

Murine clearance studies. Clearance of EMAP II in mice was assessed using ^{125}I-labelled EMAP II. EMAP II was radioiodinated by the Bolton and Hunter method (3.2 mol of ester/mol of protein) (Bolton A., 1973), and the tracer was 99% precipitable in trichloroacetic acid (20%), migrated as a single band with Mr ~20 kDa on SDS-PAGE, and had a specific radioactivity of ~8000 cpm/ng. Balb/c mice received ^{125}I-EMAP II (0.26 μg) either intravenously (IV) via the tail vein or intraperitoneally (IP). Plasma samples were taken at the indicated times, and animals were sacrificed at 24 hours. Organs were then dried, weighed and radioactivity assessed. In addition, C57BL6/J mice bearing 14 day old subcutaneous B16 tumors received ^{125}I-EMAP II (0.26 μg/animal; IP), and 1 hour before sacrifice ^{51}Cr-labelled microspheres (10 μ) in normal saline were infused (the latter to monitor residual blood in the tissue). These studies were performed to define ^{125}I-EMAP II plasma clearance, volume of distribution, and accumulation in tumor

tissue. In each case, tissue associated radioactivity was determined on weighed samples either after drying (for total radioactivity), or following homogenization of tissue and trichloroacetic acid precipitation (20%). ^{125}I-EMAP II in the tissue was corrected for residual blood based on the presence of ^{51}Cr-labelled microsperes. Plasma ^{125}I-EMAP II concentration data were fit to a two-compartment open model using nonlinear regression by extended leat squares analysis (Siphar, SIMED, Creteil, France). In order to assess the "goodness of fit," residual analysis (an examination of the standard deviation) was performed. In addition to the Likelihood test, Akaike, Leonard and Schwarz criteria were tested to select the most appropriate model (Yamoaka K., 1978). $t_{1/2}\alpha$, $t_{1/2}\beta$, $t_{1/2}r$ denote half-lives for distribution, elimination and resorption half-lives, respectively.

Tumor models. LLC and B16 (F10) cells were rinsed with Hanks buffered saline solution, trypsinized, counted, resuspended in phosphate-buffered saline, and injected subcutaneously into backs of C57BL6/J mice (2×10^6 cells/animal). On the third day following administration of tumor cells, animals underwent IP injection of EMAP II every 12 hrs for 12 days of either vehicle alone (serum albumin, 1%), vehicle + EMAP II (at 100 or 1000 ng), or vehicle + heat-inactivated EMAP II (1000 ng). Tumor growth was assessed with calipers every third day (from days 3-15), and tumor volume was calculated according to the formula for a spehircal segment (35), $V = \pi h (h^2+3a^2)/6$, where h= height of the segment, a= (length+width)/2, and V= volume. Tumor volume data was analyzed using the Kruskal-Wallis one way ANOVA and a Mann-Whitney mean rank test. Animals were sacrificed and tumors analyzed histologically at day 15.

Histologic analysis was performed on formalin fixed, paraffin embedded tissue, using hematoxylin and eosin staining. DNA nick translation was used in tumor tissue (LLC an Meth A) to evaluate apoptosis. Paraffin embedded tumor slices were deparaffinized and digoxigenin-11-UTP was

used to label fragmented DNA according to the Genius 1 kit (Amersham, location). In brief, tissue was treated with proteinase K (1 μg/ml), and incubated with digoxigenin-11-UTP, klenow, and DNTP's overnight. Nitroblue tetrazolium and alkaline phosphatase were used to reveal the digoxigenin labelled DNA fragments. In addition, sequential sections of Meth A tumors underwent analysis for DNA fragmentation and EMAP II. Apoptosis was analyzed in cultured cells exposed to EMAP II (10 and 100 ng). Cultures were exposed to hypoxia (pO_2 ≈14 torr) using a specially constructed controlled environment chamber, as described previously (Ogawa S., 1990). Cells were incubated with EMAP II, as indicated, and were then fixed in paraformaldahyde (2.5%), rinsed with phosphate-buffered saline, incubated with 6-diamidino-2-phenylindoledilactate (DAP-1; final concentration, 1 ng/ml) and mounted with glycerol (10%). DNA laddering for apoptosis in cultured cells exposed to EMAP II was performed as described (Gorczyca W., 1993). Briefly, after 12 hours of exposure to EMAP II, cultures were treated with lysis buffer (Tris-borate buffer, 45 mM; EDTA, 1mM; pH 8.0; NP-40, 0.25%), digested with proteinase K (1 mg/ml) and RNAase A (0.1 mg/ml), and then DNA was purified by phenol-chloroform extraction. DNA (10 μg/lane) was subjected to agarose gel (1.8%) electrophoresis at 40 volts using an 100 bp ladder as standard (Boehringer Mannheim). Gels were stained with ethidium bromide.

Results

Preparative scale purification of recombinant EMAP II.

Previous studies have employed FPLC Mono Q followed by preparative SDS-PAGE to prepare homogenous EMAP II (Kao J., 1992; Kao J., 1994), resulting in microgram quantities of purified EMAP II. In order to obtain the larger amounts of polypeptide necessary for a range of studies to characterize properties of EMAP II, using in vitro and in vivo systems, a larger scale approach was employed. E. coli paste, from bacteria transformed with the a plasmid expressing mature EMAP II, was disrupted using a microfluidizer, the latter

method found to be 100% effective based on light microscopy. Addition of polyethylene imine to a final concentration of 0.25% removed many of the contaminating polypeptide bands (Fig. 1, compare lanes 1 and 2) from the homogenate. Application of the polyethylene imine supernatant (800 mg) to Heparin Sepharose followed by elution with an ascending salt gradient yielded a pool of protein (150 mg) significantly enriched in EMAP II (Fig. 2A) and containing only minor contaminants by SDS-PAGE (Fig. 1, lane 3). The majority of contaminating protein eluted in the flow through of the column or at the beginning of the salt gradient (Fig. 2A). Protein yields of over 90% were obtained from the concentration and buffer exchange of the Heparin Sepharose pool.

SP Sepharose High Performance chromatography of the EMAP II-rich pool from Heparin Sepharose (130 mg) further removed contaminating proteins (Fig. 2B), shown to be principally the high molecular weight contaminants, as judged by SDS-PAGE (Fig. 1, lane 4). The latter more slowly migrating polypeptide bands were subjected to N-terminal sequence analysis and shown to be of bacterial origin and unrelated to EMAP II or the transformation procedure. EMAP II-containing fraction eluted at approximately 0.15 M sodium chloride in the gradient, and protein yields of over 90% were obtained for this step. Final purification of the SP Sepharose pool (130 mg) was achieved by Phenyl Toyopearl Chromatography (Fig. 2C) which removed any residual contaminating non-EMAP II bands (Fig. 1, lane 5). EMAP II-containing fractions eluted at approximately 1 M $(NH_4)_2SO_4$ and yields of nearly 100% were obtained.

Protein yields of over 98% were obtained by the concentration and formulation of EMAP II into phosphate-buffered saline. Posidyne filtration caused no loss of protein and reduced endoxoin levels to <10 pg/3-5 mg purified EMAP II protein. The final formulated pool was seen as an apparently diffuse band at 21 kDa by gel

electrophoresis (Fig. 1, lane 6). The faint band at Mr ~40 kDa was probably due to aggregation of EMAP II, as indicated by the characterization of the purified material below. Mass spectrometry gave measured mass of 18,006 which is close to the expected mass of 17,970. N-terminal sequence analysis showed a single sequence with an 100% match between purified murine EMAP II and the published sequence (Kao J., 1992; Kao J., 1994). The purified material was also recognized by anti-mature EMAP II amino terminal peptide IgG by immunoblotting, and in the endothelial cell tissue factor induction assay gave activities of 0.3-0.4 units/ng of protein. The latter is what has been observed with nonrecombinant EMAP II prepared from meth A-induced murine fibrosarcomas (Kao J., 1992) or recombinant EMAP II prepared by a non-preparative scale method (Kao J., 1994). The faint band at Mr ~40kDa observed on SDS-PAGE of purified (r)EMAP II preparations (Fig. 1, lane 6), was thus most likely to represent aggregates (identical N-terminal sequence and immunoreactive with anti-mature EMAP II-N-terminal peptide IgG (Kao J., 1992) on immunoblotting. Heat-treated EMAP II was boiled for 15 min, and had no activity with respect to previously described effects of EMAP II on ECs or mononuclear phagocytes (Kao J., 1992; Kao J. 1994).

Effect of EMAP II on bFGF-induced angiogenesis. To evaluate the ability of EMAP II to regulate blood vessel formation in response to known growth factor, bFGF and herapin were mixed with a gel of basement membrane proteins produced by Engelbreth-Holm-Swarm tumor cells (Matrigel) to serve as a model angiogenic stimulus (Kleinman H., 1986; Passaniti A., 1992). Subcutaneous Matrigel implants in C57BL6/J mice were evaluated 14 days after inoculation for vessel formation, cellular infiltration and hemoglobin content. Histologic analysis of the gel showed formation of vessel and white cell infiltration to be most pronounced in implants from animals treated with bFGF and heparin (Fig. 3A), which closely parallelled the appearance of implants from animals whose gel content either bFGF/herapin + vehicle (albumin) or

bFGF/herapin + heat-treated EMAP II (Fig. 3B). This induction of blood vessel formation is similar to that reported previously with bFGF in this model (Passaniti A., 1992). In contrast, implants from animals treated with bFGF/herapin + EMAP II displayed marked reduction in vessel ingrowth (Fig. 3C); little-to-no vessel formation and only a minimal cellular infiltrate was observed (n=36). Consistent with these histologic results, there was a 76% reduction in hemoglobin content in implants containing EMAP II, compared to vehicle alone (defined as 100%), heat-inactivate EMAP II (89%) or bFGF/herapin (146%) (Fig. 3D).

Plasma clearance and tissue deposition of infused EMAP II.

In order to perform in vivo studies with EMAP II, its plasma clearance and tissue deposition was evaluated (Fig. 4A). Clearance studies were performed using ^{125}I-EMAP II administered either IV or IP. The fall in plasma concentration of ^{125}I-EMAP II after IV injection fit best to a bi-exponential function; the distribution and elimination half-lives were 0.47±0.17 and 103±5 min, respectively. Following IP injection, ^{125}I-EMAP II was detected in plasma after 1 min, and the maximum concentration was reached by 35±10min. The resorption phase of EMAP II handling in vivo was best described as a first-order process. The elimination phase following IP administration fit to a monoexponential decline, and the resorption and elimination half-lives were 50.1±0.10 and 102±6 min, respectively.

Animals bearing B16 tumors were analyzed for tumor-associated radioactivity after receiving an IP injection of ^{125}I-EMAP II; at 1, 6, and 12 hours radioactivity accumulated in the tumor (56±20 cpm/mg tumor tissue; 63±8.9% precipitable in 20% trichloroacetic acid) was 2.5-fold greater than in the spleen (22.7±10 cpm/mg; 39.7±16.7% precipitable in 20 trichloroacetic acid) (Fig. 4B-C; the figure shows data from the 12 hour time point that is consistent with that observed at earlier times). Other normal tissues, such as liver and brain, also showed a lower

amount of radioactivity compared with that present in the tumor (Fig. 4C).

Effect of EMAP II on growth of primary tumors. Mice implanted subcutaneously with LLC cells developed tumors which showed a marked reduction in size when animals received EMAP II, versus controls with vehicle alone or vehicle + heat-treated EMAP II (Fig. 5A). These differences were statistically significant using either the Kruskal-Wallis one way ANOVA analysis ($p<0.034$) or by Mann-Whitney analysis ($p<0.003$) (Fig. 5A). Histologic study of LLC tumors allowed to grow for 15 days and injected every 12 hrs with vehicle (albumin 1%) demonstrated a densely packed and uniform cell population (Fig. 5B). Heat-inactivated EMAP II (at 1000 ng) was without effect on tumor histologic appearance (Fig. 5C). After administration of active EMAP II at 100 ng or 1000 ng twice daily for 12 days, a dose-dependent appearance of pyknotic bodies was observed (Fig. 5D-E), consistent with apoptosis, which appeared to parallel the course of capillaries. This was confirmed by assessing DNA fragmentation on sequential sections by end-labelling with digoxigenin-11-dUTP; compared with control tumors treated with vehicle alone, positive staining, characteristic of apoptosis, was observed in tumors treated with EMAP II (Fig. 5F-G). There was a dose-dependent increase in apoptotic areas present in the tumors with 100 and 1000 ng of EMAP II. Similar inhibition of tumor growth was observed when EMAP II was administered to mice with b16 melanomas.

These results led to an assessment of whether apparent necrosis in the perivascular areas of Meth A sarcomas, which produce EMAP II endogenously (as assessed by ELISA of meth A and tumor tissue), might be associated with apoptosis. DNA fragmentation was demonstrated by in situ nick translation in meth A, and appeared to parallel the vasculature in a pattern resembling that observed in LLC tumors in animals that received EMAP II. Pilot studies have

suggested that EMAP II was also present at these perivascular sites in the meth A tumor.

Effect of EMAP II on endothelium. EMAP II was initially isolated from Meth A tumors, due to their known thrombohemorrhage, resulting in spontaneously occurring areas of apparent necrosis/apoptosis (Old L., 1986). These data, along with examination of multiple LLC and B16 melanomas following treatment with EMAP II (in which apoptosis followed a perivascular pattern), suggested that EMAP II might modulate endothelial cell growth.

When confluent ECs were wounded and EMAP II was administered, cells at the wound edge failed to effectively migrate/proliferate and fill the wound area (Fig. 6B) compared to untreated controls (Fig. 6A). Staining with DAP-1, to visualize the chromatin, revealed the presence of apoptotic bodies, suggesting that EMAP II induced programmed cell death (Fig. 6D) versus their absence in controls (Fig. 6C). This effect was selective for rapidly growing ECs, as exposure of cultures approaching confluence to EMAP II had a small effect; there was a decrease in the mitotic rate and, at most, a 3-4 fold increase in apoptotic bodies (Fig. 6D) compared with untreated controls. EMAP II-induced apoptosis of rapidly growing ECs was further analyzed by electrophoresis for DNA fragmentation: characteristic ladder formation was observed in growing ECs exposed to EMAP II, whereas vascular smooth muscle cell DNA was unaffected.

As tumor tissue is also known for the presence of areas of local tissue hypoxia/hypoxemia (Olive P., 1992; Kalra R., 1994), whether EMAP II displays enhanced activity under oxygen deprivation was investigated. When ECs were exposed to hypoxia (pO_2 ≈14 torr) for 12 hrs, there was a decrease in mitotic rate (Fanburg B., 1987; Shreeniwas R., 1991) and a slight increase in apoptosis, which was magnified 50-fold in the presence of EMAP II.

Discussion

Formation of tumor vasculature is essential for growth and development of the neoplasm, but also provides an opportunity for therapy at the level of interrupting vascular integrity or the delivery of cytotoxic agents. Vasculature in tumors is known for its prothrombotic diathesis, increased permeability, exaggerated response to cytokines, and increased number of growing/migrating endothelial cells (Folkman, J., 1995; Old L., 1986; Asher A., 1987; Constantinidis I., 1989; Watanable N., 1988; Senger D., 1983). These properties, which distinguish vessels in the tumor bed from these in normal tissues, suggest parameters to be exploited in defining agents to selectively target tumor neovasculature.

Studies to identify EMAP II began with a characterization of mediators produced by Meth A tumor cells which perturbed properties of the endothelium (Kao J., 1992; Kao J., 1994; Nawroth P., 1988). Thus EMAP II was first studied based on its modulation of endothelial properties, such as induction of leukocyte adherence molecules and the procoagulant cofactor tissue factor. Further studies on mononuclear phagocytes and polymorphonuclear leukocytes confirmed its ability to induce cell migration and activation. These data suggested that EMAP II had properties of an inflammatory cytokine, at least based on in vitro findings. This was consistent with the capacity of EMAP II to enhance tumor thrombohemorrhage in response to TNF (Kao J., 1992; Kao J., 1994). However, the results of other in vivo experiments were not consistent with an important role for EMAP II as an inflammatory cytokine; in the footpad model, EMAP II induced only transient, mild swelling and leukocyte infiltration, and, following IV infusion, EMAP II elicited transient pulmonary leukostasis and expression of other cytokines (Interleukins 1 and 6, and tumor necrosis factor, TNF) (Kao J., 1992; Kao J., 1994). Furthermore, at the highest doses of EMAP II infused (10-50 μg/animal), there was no evidence

of severe toxicity and there were no fatalities (Kao J., 1994). This contrasts with the more potent effects of TNF under similar conditions (Beutler B., 1986). These data aroused suspicion that EMAP II might have properties distinct from proinflammatory cytokines. This supposition is supported by the finding reported herein that EMAP II had anti-angiogenic properties in vivo, in contrast to the angiogenic effects of TNF (Frater-Schroder M., 1987; Leibovich S., 1987).

In the current experiments EMAP II's anti-proliferative properties in vitro and antiangiogenic activity in vivo have been explored. Studies in the Matrigel model demonstrated reduction in neovascularization, consistent with endothelium comprising a cellular target of EMAP II. The diminished sized of tumors in the presence of EMAP II could result both from diminished neovascularization, as well as destruction of vessels already present in the tumor bed. The perivascular location of areas of apoptosis both in LLC, receiving exogenous EMAP II, and in Meth A tumors, producing EMAP II endogenously, suggests that vasculature was a target of the cytokine. The effect of EMAP II is less likely to be mediated by direct action on the tumor cells, as EMAP II does not impact adversely on tumor cell growth and viability in vitro. In contrast, experiments with cultured endothelium demonstrated induction of apoptosis of rapidly growing cultures, whereas there was a less pronounced effect on cultures approaching confluence. Although mitoses in just-confluent endothelium were markedly diminished, induction of programmed cell death was minimal, possibly to a cell cycle-dependence of EMAP II-induced cellular effects. As hypoxia is an important stimulus for angiogenesis, it was of interest to note that EMAP II had an exaggerated apoptotic effect in endothelial cultures subjected to oxygen deprivation. This was not observed in either smooth muscle cells or fibroblasts under similar hypoxic conditions. Data, showing high affinity endothelial binding sites for EMAP II, contrasted to the absence of such sites

on tumor cells, would be consistent with differential expression of EMAP II receptors on these cell types. Although the basis for this apparent specificity of EMAP II at the cellular level is at present unclear, this might reflect differences in receptor expression or post-receptor signalling. Such specificity is clearly critical for guiding future work directed at mechanisms underlying actions of EMAP II on the cellular and molecular levels.

RETINOPATHY

A hypoxia induced retinal neovascularization model has been well established by the "Association for Research in Vision and Ophthalmology Statement for the use of Animals in Ophthalmic and Vision Research," is followed. To produce retinal neovascularization, litters of 7 day old (postnatal day seven -P7) C57BL/6J mice with nursing mothers are exposed to 75% oxygen for 5 days and returned to room air at age P12 (room air will mimic hypoxia in the mouse). Animals receive IP vehicle (mouse serum albumin) - control, EMAP II 100-1000ng or heat inactivated EMAP II (1000ng) every twelve hours beginning on P7 and continuing until evaluation of retina. Mice of the same age kept in room air are used as controls. The eyes of the mice are evaluated on days P13-18 in room air for the development of retinopathy. This is accomplished by humane euthanasia of the mice, the infusion of a fluorescein-dextran solution and the use of fluorescence microscopy for the viewing of the eye vasculature. By assessing the amount of new vascularization, inhibition of retinal angiogenesis is demonstrated.

Example 2 Endothelial-monocyte Activating Polypeptide II, a Novel Anti-tumor Cytokine That Suppresses Primary and Metastatic Tumor Growth, and Induces Apoptosis in Growing Endothelial Cells

Neovascularization is essential for growth and spread of primary and metastatic tumors. From murine methylcholanthrene A-induced fibrosarcomas, well-known for their spontaneous vascular insufficiency, a novel cytokine has been identified and purified, Endothelial-Monocyte Activating Polypeptide (EMAP) II, that potently inhibits tumor growth in vivo, and appears to have anti-angiogenic activity in vivo and in vitro. Mice implanted with a matrix containing basic fibroblast growth factor showed an intense local angiogenic response which EMAP II blocked by 76% ($p<0.001$). Intraperitoneally administered recombinant EMAP II suppressed the growth of primary Lewis Lung Carcinomas, with a reduction in tumor volume of 65% compared with controls ($p<0.003$ by Mann-Whitney). In a lung metastasis model, EMAP II blocked outgrowth of Lewis lung carcinoma macrometastases; total surface metastases were suppressed by 65%, and of the 35% metastases present, about 80% of these were inhibited with maximum diameter <2 mm ($p<0.002$ compared with controls). In growing capillary endothelial cultures, EMAP II induced apoptosis in a time- and dose-dependent manner; an effect enhanced by concomitant hypoxia, whereas other cell types, such as Lewis Lung carcinoma cells, were unaffected. These data suggest that EMAP II is a tumor suppressive mediator with anti-angiogenic properties allowing it to target growing endothelium and limit establishment of neovasculature.

The following abbreviations are used herein below: meth A, methylcholanthrene A-induced fibrosarcoma; EMAP, Endothelial-Monocyte Activating Polypeptide; TNF, Tumor Necrosis Factor; EC, endothelial cell; SMC, smooth muscle cell; LPS, lipopolysaccharide; r, recombinant; BFGF, basis fibroblast growth factor; LLC, Lewis Lung Carcinoma; IV, intraperitoneal; IP, intraperitoneal; DAP-1, 6-diamidino-

2phenylindoledilactate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; VEGF, Vascular Endothelial Growth Factor.

Murine methylcholanthrene A-induced (meth A) fibrosarcomas, which exhibit spontaneous vascular insufficiency manifested by a heterogeneous pattern of thrombohemorrhage and central necrosis, as well as their failure to form metastatic lesions (Old, L., 1986; Old, L., 1961), provide an ideal starting point for isolation of tumor-derived mediators which perturb the vasculature (Clauss, M., 1990; Clauss, M., 1990; Kao, J., 1992; Kao, J., 1994). A novel cytokine-like molecule was purified, Endothelial-Monocyte Activating Polypeptide (EMAP) II, from meth A-conditioned medium based on its capacity to induce activation of endothelial cells and mononuclear phagocytes (Kao, J., 1992; Kao, J., 1994). This single chain polypeptide, devoid of a signal sequence, is initially synthesized as a ≈34 kDa intracellular precursor, which is processed to the mature ≈20 kDa form and released extracellularly by a yet to be identified pathway. EMAP II showed no significant homology to other known proteins, such as cytokines or growth factors. However, an aspartic acid residue is present in the P-1 position in both murine and human EMAP II, suggesting a cysteine protease in the Interleukin 1ß-converting enzyme family might be responsible for producing mature EMAP II from its pro-form. Our initial characterization of EMAP II suggested that its properties resembled those of proinflammatory mediators. For example, EMAP II induced endothelial release of von Willebrand factor, translocation of P-selectin to the cell surface, synthesis and expression of E-selectin and procoagulant tissue factor (Kao, J., 1992; Kao, J., 1994); these, and EMAP II-mediated activation of cultured monocytes, resulting in production of cytokines and stimulation of cell migration, suggested phlogogenic properties. However, EMAP II administered in vivo, locally or systemically, gave rise to, at most, mild and transient inflammation (Kao, J., 1994), suggesting that its effects

were quite different from those of tumor necrosis factor (TNF) or Interleukin 1 (Old, L., 1961; Sherry, B., 1988; Dinarello, C., 1993).

EMAP II has anti-angiogenic properties preventing blood vessel ingrowth in an experimental angiogenesis model, and suppressing the growth of primary and metastatic tumors without toxicity in normal organs. Consistent with this hypothesis, EMAP II appears to target growing endothelial cells; exposure of growing cultured capillary endothelium to EMAP II induces apoptosis, which is magnified by concomitant hypoxia. These data suggest that EMAP II is a polypeptide with anti-angiogenic properties which targets rapidly growing vascular beds, and suggests that, in addition to its effects on tumor neovessels, it may contribute to phases of normal development and wound repair in which cessation of blood vessel growth and tissue resorption are critical.

METHODS

Cell culture and in vitro assays. Bovine aortic and capillary endothelial cells (ECs) were isolated from calf aortae and adrenal, respectively, grown in culture and characterized, based on the presence of vonWillebrand factor and thrombomodulin, as described previously (Gerlach, H., 1989). Bovine vascular smooth muscle cells (SMCs) were prepared by additional scraping of the aortae following removal of the endothelium, and were characterized based on the presence of smooth muscle cell actin (Gown, A., 1985). Lewis Lung Carcinoma (LLC) and B16(F10) melanoma cells, obtained from American Type Culture Collection (ATCC), were maintained in high glucose-defined Minimal Essential Medium (DMEM; Gibco) containing fetal bovine serum (10%). Meth A tumor cells (Center for Cancer Research, NY) were grown as described (Old, L., 1986). EMAP II-induced apoptosis was studied in subconfluent endothelial cultures (Gerlach, H., 1989). DNA fragmentation was quantified using a 5-bromodeoxyuridine (Brdu) incorporation kit from Boehringer-Mannheim according to the manufacturer's

instructions. In brief, cells were incubated for 12 hrs with BrdU, were plated for 24 hrs on 96-well plates, and were then treated with either vehicle (fetal bovine serum, 10%) alone or vehicle + rEMAP II, as indicated. After 12 or 24 hrs at 37°C, cells were lysed, centrifuged (250xg) for 10 min, and then the top 0.1 ml was aspirated and applied to an ELISA plate with pre-adsorbed anti-DNA antibody. Site of primary antibody binding were identified using peroxidase-conjugated anti-BrdU antibody. Where indicated, ECs were incubated with rEMAP II and/or exposed to hypoxia (pO_2 ≈14 torr) using a specially constructed controlled environment chamber, as described previously (Shreeniwas, R., 1991). After the incubation period, cells were fixed in paraformaldahyde (2.5%), rinsed with phosphate-buffered saline, incubated with 6-diamidino-2-phenylindoledilactate (DAP-1; final concentration, 1 ng/ml) and mounted with glycerol (10%). F-actin was visualized in cultured cells by incubation with rhodamine-conjugated phalloidin (Molecular Probes). Wounding of endothelial monolayers was performed using a 2-mm cork borer (Selden, S., 1981).

Preparation of recombinant murine EMAP II , and detection of EMAP II transcripts and antigen. Recombinant EMAP II was prepared from E. coli (host HMS174[DE3]) transformed with a plasmid containing the coding sequence for mature EMAP II, as described previously (Kao, J., 1994). Frozen (-80°C) E. coli cell paste was mixed 1:10 (w/v) with Tris-HCl (20 mM; pH 7.4) containing octyl-ß-glucoside (0.1%) and an homogeneous suspension was formed by agitation using a microfluidizer for 20 min (speed 60) at 4°C. Polyethylene imine at pH 7 was then added to the homogenate to a concentration of 0.25%, solids were removed by centrifugation (5000xg; 30 min), and the supernatant was retained. After filtration (0.2 μm), the sample was applied (3 mls sample/ml of gel) to Heparin Sepharose CL-4B (Pharmacia; 120 ml bed volume) equilibrated in Tris-HCl (20 mM; pH 7.4) containing octyl-ß-glucoside (0.1%), and the column was eluted with a linear ascending NaCl gradient.

Fractions were pooled on the basis of purity by silver stained SDS-PAGE, by immunoblotting with antibodies prepared to the N-terminus of mature EMAP II, and by biological activity measured in a tissue factor induction assay (Kao, J., 1994). The Heparin Sepharose pool was concentrated using an Amicon Stirred Cell (Amicon), the retentate was desalted into 3-(Morpholino)-propane-sulfonic acid (MOPS, 25 mM; pH 6.9), and was then applied to an SP Sepharose High Performance (Pharmacia) cation exchange column (55 ml bed volume). The column was eluted by application of a 0 to 0.5 M ascending linear salt gradient in MOPS, and EMAP II-containing fractions were adjusted to 2 M in $(NH_4)_2SO_4$, applied to a Phenyl Toyopearl 650 M (Tosohaas) column (90 ml bed volume), equilibrated in sodium phosphate (20 mM; pH 7) containing 1 M $(NH_4)_2SO_4$. The column was eluted with a descending salt gradient (2 to 0 M) in sodium phosphate (20 mM), and EMAP II in the Phenyl Toyopearl column eluate was concentrated to 3-5 mg/ml, and formulated into phosphate-buffered saline (PBS; pH 7.4) by buffer exchange on a Sephadex G25 column (as above). Lipopolysaccharide (LPS) was removed using filtration through a Posidyne filter (Pall Corp.), and LPS levels were estimated using the Endospecy chromogenic assay (limit of detection <10 pg/ml). Purified EMAP II was subjected to N-terminal sequence analysis, mass spectrometry and SDS-PAGE; the current material was found to be homogeneous according to these criteria. The phenyl-toyopearl column and Posidyne filtration steps appeared to remove certain toxic contaminant(s) associated with rEMAP II prepared by preparative electrophoresis in previous studies (Kao, J., 1994).

Antibody to rEMAP II was prepared by standard methods in rabbits (Vaitukatis, J., 1981) and was found to be monospecific, based on immunoblotting of plasma and cell extracts, and anti-EMAP II IgG blocked the activity of rEMAP II in cell culture assays (Kao, J., 1994). This antibody was used to develop an ELISA to detect EMAP II antigen by

the general protocol described previously (Kao, J., 1994).

PCR analysis for EMAP II transcripts employed RNA extracted from murine tissues (Balb/c mice) using the RNA Stat-60 kit (Teltest) according to the manufacturer's instructions, and reverse transcribed (1 μg) using Taq polymerase (Perkin-Elmer-Cetus). Primers were used for EMAP II (#1: GCATCGCGTCTGGATCTTCGAATT ; and, #2: GTATGTGGCCACACACTCAGCATT) and ß-actin (Gibco). Thermocycling parameters for the experiment shown in Fig. 7 were: 94°C for 30 sec; 55°C for 30 sec; and, 72°C for 30 sec for a total of 35 cycles. Samples were subjected to agarose gel (1%) electrophoresis and bands were visualized by ethidium bromide staining. Identity of amplicons was confirmed by Southern blotting with the appropriate cDNA probes.

Matrigel model. Matrigel (Kleinman, H., 1986; Passaniti, A., 1992)(Collaborative Research) containing either vehicle (1% BSA), rEMAP II (100 ng/ml) + vehicle; basic Fibroblast Growth Factor (bFGF; 100 ng/ml; Collaborative Research) + heparin (40 U/ml; Sigma) + vehicle; rEMAP II (100 ng/ml) + bFGF/heparin + vehicle, or heat-inactivated rEMAP II (100 ng/ml; alone or with bFGF/heparin) + vehicle was mixed at 4^0 C. Matrigel mixtures were injected subcutaneously into C57BL6/J mice (0.25 ml/site) at two sites per animal. The angiogenic response was analyzed at 7 and 14 days post-inoculation by routine histology and hemoglobin assay (Sigma).

Murine clearance studies. Clearance of EMAP II in mice was assessed using ^{125}I-labelled rEMAP II. rEMAP II was radioiodinated by the Bolton and Hunter method (3.2 mol of ester/mol of protein; 16), and the tracer was 99% precipitable in trichloroacetic acid (20%), migrated as a single band with Mr ≈20 kDa on SDS-PAGE, and had a specific radioactivity of ≈8000 cpm/ng. Balb/c mice received ^{125}I-rEMAP II (0.26 μg) either intravenously (IV) via the

tail vein or intraperitoneally (IP). Plasma samples were taken, and animals were sacrificed at 24 hours. Organs were then dried, weighed and radioactivity assessed. In addition, C57BL6/J mice bearing 14 day old subcutaneous B16 tumors received ^{125}I-rEMAP II (0.26 μg/animal; IP), and, 1 hour before sacrifice, were infused with ^{51}Cr-labelled microspheres (10 μ) in normal saline (the latter to monitor residual blood in the tissue). These studies were performed to define ^{125}I-rEMAP II plasma clearance, volume of distribution, and accumulation in tumor tissue. In each case, tissue associated radioactivity was determined on weighed samples either after drying (for total radioactivity), or following homogenization of tissue and trichloroacetic acid precipitation (20%). ^{125}I-rEMAP II in the tissue was corrected for residual blood based on the presence of ^{51}Cr-labelled microspheres. Plasma ^{125}I-rEMAP II concentration data were fit to a two-compartment open model using nonlinear regression by extended least squares analysis (Siphar, SIMED, Creteil, France). In order to assess the "goodness of fit," residual analysis (an examination of the standard deviation) was performed (Yamoaka, K., 1978).

Murine tumor models. *To test the effect of anti-EMAP II IgG on apoptosis in meth A tumors*, mice were subcutaneously injected with meth A cells and on day 9 started on a course of IP injections every third day of either nonimmune rabbit IgG (400 μg/dose) or rabbit anti-murine EMAP II IgG (200 or 400 μg/dose). This regimen of IgG administration was based on pilot studies in which ^{125}I-rabbit anti-EMAP II IgG infused into mice demonstrated a half-life of elimination of 29.4±2.67 hrs. Animals were sacrificed at day 14 and tissue was analyzed for evidence of apoptosis as described below.

For producing primary tumors to test the effects of EMAP II treatment, LLC and B16(F10) melanoma cells were rinsed with Hanks buffered saline solution, trypsinized, counted,

resuspended in phosphate-buffered saline, and injected subcutaneously into backs of C57BL6/J mice ($2x10^6$ cells/animal). On the third day following administration of tumor cells, the tumor was reproducibly measurable, and this tumor volume was taken for comparison with later measurements of that tumor. Animals then underwent IP injection every 12 hrs for 12 days of either vehicle alone (serum albumin, 1%), vehicle + rEMAP II (at 100 or 1000 ng), or vehicle + heat-inactivated rEMAP II (1000 ng). Tumor growth was assessed with calipers every third day (from days 3-15), and tumor volume was calculated according to the formula for a spherical segment (18), $V=\pi h(h^2+3a^2)/6$, where h= height of the segment, a= (length+width)/2, and V= volume (each tumor was compared with itself over multiple measurements and change in volume was noted). Tumor volume data were analyzed using the Kruskal-Wallis one way ANOVA and a Mann-Whitney mean rank test. Data is expressed as a dimensionless ratio of observed tumor volume divided by initial (day 3) tumor volume. Animals were sacrificed and tumors analyzed histologically at day 15.

For the metastatic tumor model (O'Reilly, M., 1994; Holmgren, L., 1995), C57BL6/J mice received LLC cells subcutaneously and were observed until tumor volume reached ≥ 1.5 cm^3. Animals then received rEMAP II (1000 ng/dose) in vehicle or vehicle alone IP every 12 hrs for 72 hrs prior to resection of the primary tumor. Following complete resection of the tumor (with no recurrence), mice were observed for an additional 15 days, during which time they received rEMAP II (1000 ng IP every 12 hrs) in vehicle or vehicle alone (same schedule). On day 15, lungs were injected intratracheally with India ink (15%) to visualize lung surface nodules, and tissue was fixed in Fekete's solution (70% alcohol; 5% glacial acetic acid; 3.7% formaldehyde). Surface metastatic lesions were counted by gross inspection of the tissue under 4X-magnification, and macrometastases were defined based on a smallest surface nodule diameter >2 mm.

Tissue analysis: histology, apoptosis, immunohistology. Histologic analysis was performed on formalin fixed, paraffin-embedded tissue, using hematoxylin and eosin staining. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was used to evaluate apoptosis; paraffin embedded tumor slices were deparaffinized and digoxigenin-11-dUTP was used to label fragmented DNA according to the Genius 1 kit (Amersham). In brief, tissue was treated with proteinase K (1 μg/ml), and incubated with digoxigenin-11d-UTP, klenow, and dNTP's overnight. Nitroblue tetrazolium and alkaline phosphatase were used to reveal the digoxigenin labelled DNA fragments. Where indicated, sections for the TUNEL assay were counterstained with eosin. For immunolocalization of EMAP II and thrombomodulin antigens, rabbit anti-rEMAP II IgG was employed (5 μg/ml) and rabbit anti-murine thrombomodulin IgG (2.5 μg/ml). Tissues, fixed as above, were incubated with primary antibody for 2 hr at 37°C or 1 hr at room temperature, respectively; sites of primary antibody binding were visualized using the ABC Elite kit (Vector), and revealed by reaction with 3,3'-diaminobenzidine.

RESULTS

Effect of anti-EMAP II IgG on meth A tumors. A heterogeneous picture of vascular insufficiency is commonly observed in meth A tumors shortly after the primary tumor becomes established (Old, L., 1986; Old, L., 1961). Prior to gross loss of tumor cell viability, pyknotic changes are evident in a perivascular distribution. At higher magnification, such pyknotic areas showed evidence of DNA fragmentation, based on TUNEL assay, and their general association with vasculature was confirmed by colocalization with the endothelial marker thrombomodulin (though regions of pyknosis/apoptosis extended beyond that in proximity to the blood vessel). Immunohistologic localization of EMAP II in meth A tumors showed it be associated with the vessel wall, possibly reflecting interaction with extracellular

matrix due to its heparin-binding properties. Furthermore, it appeared that expression of EMAP II protein in the tumor was evident by day 9 and continued to increase thereafter, which corresponds to the appearance of apoptotic changes in the tumor bed. Consistent with an association of EMAP II with meth A-associated apoptosis, the presence of such lesions was markedly diminished in mice treated with a high dose of anti-EMAP II IgG.

Distribution of EMAP II in normal mice. These data suggested that EMAP II could impact on tumor viability, which led to an examination of its sequestration in normal tissues. EMAP II transcripts were demonstrated in a range of organs (brain, liver, lung, spleen, heart, kidney, smooth muscle; Fig. 7), though their levels appeared to be quite low, requiring at least 35 cycles of PCR amplification to visualize the appropriate size amplicon. This impression was confirmed by Northern analysis, which showed a low intensity band at ≈1.1 kb in meth A cell RNA (corresponding to the size of the murine EMAP II mRNA; Kao, J., 1992), not observed in the normal organs. Expression of EMAP II transcripts was unaffected by infusion of lipopolysaccharide (LPS; 100 μg/animal) or induction of hind limb ischemia. ELISA for EMAP II antigen showed virtually undetectable levels in the above normal tissues (limit of detection <250 pg/ml) and no peak of EMAP II in the plasma after LPS administration or hind limb ischemia. These data indicated that EMAP II is expressed only at the lowest levels in normal mice, and that it is unlikely to be an early mediator of the host response to acute stimuli, such as LPS or ischemia. This clearly contrasts with the rapid production and significant roles for proinflammatory cytokines such as Interleukin 1 and TNF in the acute response to tissue injury (Old, L., 1961; Sherry, B., 1988; Dinarello, C., 1993).

Preparative scale purification of recombinant EMAP II. In order to further study the effects of EMAP II in vitro and in vivo, it was important to develop a preparative scale

purification procedure. Previously, material eluted from SDS-PAGE corresponding to Mr ~20 kDa was employed. Although this material was highly purified, it was difficulat to scale-up such a method and the biologic properties of the resulting EMAP II were somewhat variable, probably due to differing degrees of denaturation/renaturaton during SDS-PAGE and gel elution. This led to development of an alternate purification strategy. Recombinant (r) EMAP II was expressed in E. coli, and purified by polyethylene imine precipitation followed by sequential application to Heparin Sepharose, SP Sepharose, and Phenyl Toyopearl. Posidyne filtration was then performed to remove LPS (levels were <10 pg at rEMAP II concentrations of 3-5 mg/ml). Details of chromatographic steps are described under Methods. The final formulated material was homogeneous on SDS-PAGE, migrating as a diffuse band at ~21 kDa. Mass spectrometry gave a measured mass of 18,006 which is close to the expected mass of 17,970. N-terminal sequence analysis showed a single sequence with an 100% match between purified murine EMAP II and the published sequence (Kao, J., 1992; Kao, J., 1994).

Effect of EMAP II on bFGF-induced angiogenesis. To evaluate the ability of EMAP II to regulate blood vessel formation in response to known growth factors, bFGF and heparin were mixed with a gel of basement membrane proteins produced by Engelbreth-Holm-Swarm tumor cells (Matrigel) to serve as a model angiogenic stimulus (Kleinman, H., 1986; Passaniti, A., 1992). Subcutaneous Matrigel implants in C57BL6/J mice were evaluated 14 days after inoculation for vessel formation, cellular infiltration and hemoglobin content. Histologic analysis of the gel showed formation of vessels to be most pronounced and comparable in implants from animals treated with either bFGF/heparin + vehicle (albumin) or bFGF/heparin + heat-inactivated rEMAP II + vehicle; higher magnification confirmed the presence of neovessels in these implants. This induction of blood vessel formation is similar to that reported previously with bFGF in this model

(Passaniti, A., 1992). In contrast, in implants from animals treated with bFGF/heparin + active rEMAP II, there was marked reduction of vessel ingrowth; little-to-no vessel formation (n=40; this experiment was repeated seven times with similar results). Consistent with these histologic findings, there was a 76% reduction in hemoglobin content in corresponding implants containing bFGF/heparin + rEMAP II, compared to bFGF/heparin + vehicle or heat-inactivated EMAP II + bFGF/heparin +vehicle (see Figs. 3 A-E).

Plasma clearance and tissue deposition of infused rEMAP II. In order to perform in vivo studies with rEMAP II, its plasma clearance and tissue deposition were evaluated. Clearance studies were performed using either intravenously (IV) or intraperitoneally (IP) administered ^{125}I-rEMAP II (Fig. 4A-C). The fall in plasma concentration of ^{125}I-rEMAP II after IV injection fit best to a bi-exponential function (Yamoaka et al., 1978); the distribution and elimination half-lives were 0.47±0.17 and 103±5 min, respectively. Following IP injection, ^{125}I-EMAP II was detected in plasma after 1 min, and the maximum concentration was reached by 35±10 min. The resorption phase of rEMAP II handling in vivo was best described as a first-order process. The elimination phase following IP administration fit to a monoexponential decline, and the resorption and elimination half-lives were 50.1±0.1 and 102±6 min, respectively. Animals bearing B16 tumors were analyzed for tumor-associated radioactivity after receiving an IP injection of ^{125}I-rEMAP II; at 1, 6, and 12 hours radioactivity accumulated in the tumor was ≈7-fold greater than in the liver, and tumor selectivity was even more pronounced in other organs. Furthermore, the precipitability of the tracer in trichloroacetic acid (20%) was greater in the tumor compared with other tissues, consistent with a relative accumulation of apparently intact rEMAP II in tumor tissue.

Effect of rEMAP II on growth of primary and metastatic

tumors. Mice implanted subcutaneously with LLC cells developed tumors (the latter do not express detectable EMAP II protein), which were first measured when they achieved a volume of about 9-10 mm^3, ~3 days following inoculation of cells. The volume of each tumor was then measured every third day, and compared with the initial volume of that tumor on day 3. Compared with tumor-bearing animals treated with vehicle alone or vehicle + heat-inactivated EMAP II, mice receiving active rEMAP II showed a striking reduction in tumor volume (Fig. 5A-G) Differences between tumor volume in control and EMAP II-treated animals were statistically significant using either the Kruskal-Wallis one way ANOVA analysis ($p<0.034$) or comparing control versus high dose rEMAP II by Mann-Whitney analysis ($p<0.003$). Histologic study of LLC tumors allowed to grow for 15 days and injected IP every 12 hrs with vehicle (albumin, 1%) demonstrated a densely packed and uniform cell population. Heat-inactivated rEMAP II (at 1000 ng/dose) was similar in appearance to the latter vehicle controls. After administration of rEMAP II at 1000 ng/dose twice daily for 12 days, areas of pyknosis were observed. At higher magnification, rEMAP II-induced areas of pyknosis had a general perivascular distribution, though pyknotic cells often extended beyond the vasculature. In Fig. %F-G, a site with several microvessels is visualized by staining for thrombomodulin, and evaluation of an adjacent section demonstrates DNA fragmentation using the TUNEL assay. There were no such apoptotic areas in control tumors treated with vehicle alone. There was a dose-dependent increase in apoptotic areas present in the tumors with 100 and 1000 ng of EMAP II. Similar inhibition of tumor growth was observed when EMAP II was administered to mice with primary B16 melanomas. Mice treated with rEMAP II were normally active, continued food/water consumption, and maintained their weights comparably to control mice.

As established metastatic foci require blood vessel ingrowth to expand beyond 1-2 mm (Fidler, I., 1994; Folkman, J.,

1989; Folkman, J., 1995; Murray, C., 1995), we reasoned that rEMAP II might suppress growth of metastatic lesions. The LLC model was employed by allowing primary tumors to grow to a volume of ≥1.5 cm^3, at which time metastases are present (but suppressed by the primary the primary tumor; O'Reilly, M., 1994; Holmgren, L., 1995). Then the primary lesion was resected (with no recurrence at the site of resection), and analysis of surface lung nodules was undertaken 15 days later. rEMAP II treatment was begun 72 hr prior to resection of the primary tumor and was continued through the end of the experiment (See Figs. 8A-E). Animals receiving rEMAP II (1000 ng IP every 12 hrs) showed significantly fewer and smaller surface nodules, compared with vehicle by gross inspection and histologic study. Consistent with these data, rEMAP II-treated animals demonstrated 65% suppression ($p<0.009$ by Mann Whitney) in outgrowth of the total number of surface metastases, compared with mice receiving vehicle alone (Fig. 8E). Of the 35% of metastases present in rEMAP II-treated animals, ≈80% of these metastases were inhibited, such that the maximum diameter was <2 mm (i.e., predominately micrometastases were present), compared with controls, ($p<0.002$ by student t-test; Fig. 8E; inset).

Effect of rEMAP II on endothelium. The data thus far demonstrated an association of EMAP II with spontaneous vascular insufficiency (meth A tumors) and with induction of apoptosis in tumors, the latter, at least in part in a perivascular distribution. These data suggested the possibility that tumor vasculature might be a target of EMAP II. To begin to assess whether rEMAP II selectively affects growing/migratory endothelium, confluent cultures were wounded and rEMAP II was added; cells at the wound edge failed to effectively migrate/proliferate and fill the wound area (Fig. 6B) compared to untreated controls (Fig. 6A). Staining with DAP-1, to visualize the chromatin, revealed the presence of apoptotic bodies localized to the wound edge (i.e., growing/migratory endothelium) in rEMAP II-treated

cultures, suggesting that rEMAP II induced programmed cell death only in this cell population, whereas confluent endothelium distal from the wound edge showed no significant effect of rEMAP II (Fig. 6D; arrows denote apoptotic bodies). Control cultures showed no such apoptotic areas (Fig. 6C). ELISA for DNA fragmentation was performed to more precisely delineate apoptotic effects of rEMAP II on endothelium: there was a dose-dependent increase in DNA fragmentation in cultured capillary endothelium, reaching 250% over that observed in controls within 24 hrs (Fig. 6E). As tumor tissue is also known for the presence of areas of local tissue hypoxia/hypoxemia (Olive, P., 1992; Kalra, R., 1994), it was assessed whether rEMAP II might display enhanced activity under oxygen deprivation. When cultured subconfluent endothelial cells were exposed to hypoxia (pO_2 ≈14 torr), DNA fragmentation was accelerated, reaching a level of 250% above that observed with vehicle alone within 12 hrs (rather than the 24 hrs required for an effect of this magnitude in normoxia). This was consistent with the accelerated appearance of apoptotic bodies by DAP-1 staining of hypoxic endothelial cultures exposed to rEMAP II. In contrast, cultured meth A fibrosarcoma, Lewis Lung carcinoma, and nontransformed vascular smooth muscle cells demonstrated no increase in DNA fragmentation after exposure to rEMAP II under the conditions above by ELISA (Fig. 6F) or DAP-1 staining.

DISCUSSION

Neovascularization is a critical regulator of the growth of both primary and metastatic neoplasms (Fidler, I., 1994; Folkman, J., 1989; Folkman, J., 1995; Murray, C., 1995). Earlier studies called attention to the role of angiogenic factors, such as vascular endothelial growth factor (VEGF; Plate, K., 1992; Warren, R., 1995; Kim, J., 1993), acidic fibroblast growth factor (Maciag, T., 1984); basic fibroblast growth factor (bFGF; Shing, Y., 1984), and angiogenin (Fett, J., 1985; King, T., 1991; Olson, K., 1994), in promoting tumor growth and establishing

metastases. For example, in a transgenic murine model, a switch in phenotype from pancreatic adenoma to malignancy was closely tied to expression of angiogenic mediators (Kandel, J., 1991), and antibody to VEGF inhibited growth of explanted human tumors in athymic mice (Warren, R., 1995; Kim, J., 1993). Similar inhibition of experimental tumor growth has also been observed with antibodies to angiogenin (Olson, K., 1994) and bFGF (Hori, A., 1991). Alternatively, recent work has identified endogenous peptides with anti-angiogenic activities, including angiostatin (O'Reilly, M., 1994), thrombospondin (Dameron, K., 1994) and glioma-derived angiogenesis inhibitory factor (Van Meir, E., 1994). They can inhibit tumor growth either at the primary tumor site (thrombospondin; Dameron, K., 1994) or at a site of distant metastases (angiostatin; O'Reilly, M., 1994; O'Reilly, M., 1996). Formation of the tumor vascular bed, as well as blood vessel formation in other situations, such as in ischemia, wound healing and atherosclerosis (Shweiki, D., 1992; Knighton, D., 1983; Kuwabara, K., 1995; Brogi, E., 1993), is presumably also controlled by the interaction of such positive and negative stimuli on endothelium in diverse vascular beds.

Carcinogen-induced murine meth A and similar tumors (Old, L., 1986; Old, L., 1961) are ideally suited to the analysis of host-tumor interactions because short-term vascular insufficiency (exaggerated by concomitant administration of an agent such as TNF), and longer-term immunologic mechanisms limit local tumor growth (Old, L., 1986; Old, L., 1961; Nawroth, P., 1988; Watanabe, N., 1988; Freudenberg, N., 1984; North, R., 1988). In fact, acute local (intratumor) administration of EMAP II to meth A tumors resulted in thrombohemorrhage in the tumor bed (Kao, J., 1994), a finding quite distinct from what we observed in the current study in which EMAP II was administered systemically at lower doses over longer times. The association of meth A-derived EMAP II with apoptosis in the tumor bed (the latter suppressed by anti-EMAP II IgG) and

immunolocalization of the polypeptide to vascular and perivascular areas of the tumor, suggested a role for this cytokine in vascular dysfunction associated with meth A tumors. Consistent with the ability of EMAP II to modulate vessel growth and/or integrity was the observation that neovessel formation into bFGF-containing implants was blocked by rEMAP II. In contrast to these results with rEMAP II, other cytokines such as transforming growth factor-ß or TNF-a have been found to induce vascular ingrowth in angiogenesis models (Leibovich, S., 1987; Fraker-Schroder, M., 1987; Madri, J., 1992).

In the LLC and B16 melanoma models, rEMAP II attenuated growth of primary tumors and resulted in a histologic picture of apoptotic tissue injury, at least in part in a perivascular distribution (similar to that seen with Meth A tumors), which progressed to nonviable tumor, probably as a result of severe ischemia . These data suggested that EMAP II was initially targeting the vasculature, leading to speculation that its tumor-suppressive effects might extend to a range of neoplasms. In support of this hypothesis, studies have shown rEMAP II to markedly attenuate growth of a human breast carcinoma line (MDA-MB 468) grown in nude mice and also to suppress C6 gliomas in rats. The observation that EMAP II diminished lung surface metastases, and, especially, macrometastases, is also consistent with the concept that neovasculature feeding the tumor, as well as in the tumor, are targets of EMAP II. It is notable that despite a prolonged course of rEMAP II treatment, ≈2 wks, no untoward effects on general health of the animals was observed, and pathologic analysis of normal organs revealed no lesions. This suggested that actions of EMAP II were localized, under these conditions, to the tumor. The data, however, do not rule out the possbility that EMAP II may have other effects on the tumor beyond that on the vasculature. For example, the action of EMAP II on endothelium or other elements in the tumor microenvironment might release diffusible mediators toxic for tumor cells,

thus causing tumor injury initially close to the vasculature, but then extending deeper into the tumor.

A salient feature of tumor vasculature, which distinguishes vessels in the tumor stroma from those in normal tissue, is the increased fraction of growing/migrating endothelial cells (Fidler, I., 1994; Folkman, J., 1989; Folkman, J., 1995). Studies in cell culture suggested a selective effect of rEMAP II on growing/migratory endothelium; cells at the leading edge of a wound in the monolayer failed to effectively fill the gap and cell proliferation was suppressed. The predominate affect appeared to be induction of apoptosis, especially in the actively dividing cell population. In contrast, postconfluent endothelium at a distance from the wound was not affected by rEMAP II. Furthermore, addition of the cytokine to cultures of growing tumor cells (LLC, B16 melanoma or meth A) showed no change in cell proliferation or induction of apoptosis, though rEMAP II suppressed these tumors in vivo. Enhanced EMAP II-induced apoptosis in hypoxic endothelial cultures provided further support for the relevance of our finding to tumor biology, as the presence of hypoxic areas in tumors is well-established (Olive, P., 1992; Kalra, R., 1994). On a cellular level, hypoxia could potentially sensitize endothelium to EMAP II by several mechanisms, including arrest of cells at the G1/S interface (Shreeniwas, R., 1991) or increased sensitivity to subsequent encounters with oxidizing stimuli. In support of the latter hypothesis, pilot studies suggest that EMAP II has an important effect on cellular redox status as addition of N-acetylcysteine blocks EMAP II-mediated endothelial apoptosis. Analysis of mechanisms through which EMAP II induces possible cellular oxidant stress, as well as elucidation of the cell surface receptor for EMAP II, will provide more definitive answers to questions concerning the specificity and selectivity of its cellular effects.

The striking feature of the in vivo studies is the

suppressive effect of rEMAP II on tumors without, apparently, an adverse affect on the function of normal organs. This may be due to EMAP II's effect on the endothelium; EMAP II could perturb endothelium in vivo not only by direct effects on endothelial apoptosis, but also by other means. For example, EMAP II-mediated induction of endothelial tissue factor could trigger local activation of clotting in the tumor bed, thereby diminishing blood flow and enlarging the volume of tumor at risk for ischemia. EMAP II might also modulate the expression of other mediators which control the local angiogenic balance, including enhanced activity of pathways regulating production of angiostatic peptides, such as angiostatin or thrombospondin, and/or might suppress expression of pro-angiogenic factors in the tumor bed. Furthermore, EMAP II might elicit endothelial production of mediators which directly impair tumor cell viability (as mentioned above). Though there are many mechanistic, physiologic and practical questions to be explored in future studies (Will EMAP II affect well-established vessels in human tumors which grow over much longer times than the accelerated murine models? Will an optimal anti-tumor regimen of EMAP II induce tumor regression or will it just be static? etc.), the data support the potential of EMAP II, a cytokine with apparent anti-angiogenic properties, to suppress primary and metastatic tumor growth, and to induce apoptosis in the tumor without apparent adverse affects on normal organs.

Example 3:

ENDOTHELIAL-MONOCYTE ACTIVATING POLYPEPTIDE II SUPPRESSES GROWTH OF C6 GLIOMAS BY TARGETING THE VASCULATURE

Endothelial-Monocyte Activating Polypeptide (EMAP) II is a novel mediator initially purified from methylcholanthrene A-induced fibrosarcomas, well-known for spontaneous vascular insufficiency and thrombohemorrhage. Testing the effect of EMAP II on C6 gliomas which elicit a characteristic angiogenic response, largely due to expression of Vascular Endothelial Growth Factor (VEGF) was therefore carried out.

Recombinant (r) EMAP II suppressed intracranial growth of C6 glioma cells implanted in rat frontal lobes by 65% ($p<0.003$), with residual tumor volume composed predominately of apoptotic cells (≈80%). Similarly, rEMAP II had a striking effect on C6 gliomas grown subcutaneously in nude mice, causing a six-fold decrease in tumor volume, without evidence of systemic toxicity. rEMAP II blocked the angiogenic response to locally administered VEGF, demonstrating a direct effect of EMAP II on VEGF-driven vascular ingrowth. Ultrastructural study of tumor vasculature from animals treated with rEMAP II showed intravascular accumulation of platelets and fibrin, as well as findings consistent with apoptosis of the endothelium. Consistent with the ability of EMAP II to target the vasculature, rEMAP II induced apoptosis and bound specifically (K_d ≈xx nM) to growing cultures of human umbilical vein endothelial cells, whereas it had no affect and displayed no specific binding to C6 glioma cells. These studies demonstrate that EMAP II has anti-tumor activity on C6 gliomas, at least in part, through its affect on the vasculature, and suggest its possible application to a range of solid tumors.

The following abbreviation are used herein below: VEGF, Vascular Endothelial Growth Factor; EMAP, Endothelial Monocyte Activating Polypeptide II; r, recombinant; IP, Intraperitoneal; IT, Intratumoral; TUNEL, Deoxynucleotidyl Transferase-mediated DUTP-biotin nick end labelling.

Vascularization of solid tumors is critical for their growth beyond a small collection of neoplastic cells (Fidler, I., 1994; Folkman, J., 1989; Folkman, J., 1995). In the central nervous system, in which vasculature is insulated from the neuronal compartment by the blood-brain barrier, effective mechanisms for induction of neovasculature have evolved to support tumor growth. Glioblastoma, the most frequently occurring intracranial neoplasm, displays characteristic vascularization with evidence of endothelial proliferation

and a complex vascular network, thereby providing an especially relevant example of ongoing angiogenesis (San Galli, F., 1989; Plate, K., 1992; Wesseling, P., 1994; Plate, K., 1995). Although induction of vascular ingrowth by glioblastomas is likely to involve multiple mediators, studies with patient-derived glioblastoma multiforme and rat gliomas have emphasized the contribution of Vascular Endothelial Growth Factor (VEGF) (Plate, K., 1992; Shweiki, D. 1992; Weindel, K., 1994; Plate, K., 1994; Samoto, K., 1994). The secreted isoform of VEGF (residues 1-165) is produced by glioblastoma/glioma at the tumor margin, especially at sites of local necrosis (and presumably, hypoxia), enhancing neovessel formation by attracting endothelium which has been shown to selectively express the VEGF receptor Flk-1 (Plate, K., 1992; Shweiki, D. 1992; Weindel, K., 1994; Plate, K., 1994; Samoto, K., 1995). Direct evidence of a role for VEGF in glioma growth derives from experiments demonstrating that antibodies to VEGF (Kim, K., 1993), a dominant negative mutant of Flk-1/VEGF (Millauer, B., 1994), and VEGF antisense introduced into gliomas suppresses the tumors (Saleh, M., 1996).

VEGF has emerged as an angiogenic factor involved in physiologic and pathophysiologic vascular responses (Houck, K., 1991; Keck, P., 1989). This polypeptide was initially characterized based on its ability to increase vascular permeability when injected subcutaneously into guinea pigs (Keck, P., 1989). In this context, VEGF has been show to have other properties associated with inflammatory mediators in vitro, including induction of the procoagulant tissue factor on endothelial cells and mononuclear phagocytes (Clauss, M., 1990). Although the relevance of these findings to the biology of VEGF in vivo has not been clarified, it has been speculated that this could account for pathologic findings in the vasculature of gliomas, including evidence of vascular leakage and local thrombi. The role of VEGF as a central angiogenic mediator has been demonstrated more directly. Deletion of the VEGF gene

results in an embryonic lethal, with failure of vasculogenesis (Harpal, K., 1996). In pathophysiologic situations, VEGF has been implicated in neovascularization associated with diabetic retinopathy, ischemic events, and tumor growth (Shweiki, D. 1992; Weindel, K., 1994; Plate, K., 1994; Samoto, K., 1994; Miller, J., 1994; Aiello, L., 1994).

Endothelial-Monocyte Activating Polypeptide (EMAP) II is a novel mediator initially identified in meth A tumors, well-known for their spontaneous vascular insufficiency (Kao, J., 1992; Kao, J., 1994). Acute administration of EMAP II directly into tumors elicited thrombohemorrhage and sensitized tumor vasculature to subsequent systemic infusion of tumor necrosis factor. Treatment of mice bearing Lewis Lung Carcinomas or B16 melanomas with low concentrations of EMAP II administered systemically for several weeks resulted in tumor regression and a pathologic picture of patchy apoptosis apparently radiating from tumor vasculature (Schwarz, M., 1995). These findings, along with the ability of EMAP II to inhibit vascular ingrowth elicited by implants impregnated with basic fibroblast growth factor and its lack of a direct cytotoxic/cytostatic effect on tumors cells, suggested that EMAP II might target tumor vasculature. The goal of the studies was to determine if EMAP II could antagonize the angiogenic effects of glioma-derived angiogenic factors, especially VEGF, thereby implying its potential to block pathologic vascular ingrowth. The results herein indicate that systemically administered EMAP II blocks neovessel formation in response to VEGF in a Matrigel model, and that it potently suppresses growth of C6 gliomas. Ultrastructural studies revealed that EMAP II induced early changes in the vasculature including intravascular fibrin formation, deposition of platelets, and findings consistent with endothelial apoptosis. This was consistent with the results of in vitro functional and binding studies which indicated that endothelial cells, rather than C6 glioma cells, were the target of EMAP II.

MATERIALS AND METHODS

Cell culture and preparation of recombinant (r) EMAP II. C6 glioma cells (Benda, P., 1971) were obtained from ATCC and were grown in Dulbecco's Modified Eagle Medium containing fetal bovine serum (10%; Gemini, Gibco, Grand Island NY). Mouse brain endothelial cells were characterized and grown as described (Gumkowski, F., 1987). Human umbilical vein endothelial cells were grown and characterized as described (Kao, J., 1992). DNA fragmentation was evaluated by agarose gel electrophoresis (xBorczyca et al., 1993- ask dave p.). Radioligand binding studies employed ^{125}I-rEMAP II and cultured C6 glioma or endothelial cells. rEMAP II was radiolabelled by the Bolton and Hunter method (Bolton, A., 1973); the tracer was >95% precipitable in trichloroacetic acid (20%) and migrated as a single band with Mr ≈20 kDa on SDS-PAGE. rEMAP II was prepared from E. coli transformed with a plasmid containing the coding sequece for mature murine EMAP II, and was purified by a modification of our previous procedure (Kao, J., 1994) using sequential chromatography on Heparin Sepharose, SP Sepharose, and Phenyl Toyopearl chromatography, followed by filtration through a Posidyne filter to remove lipopoysaccharide. The final material was migrated as a single band on SDS-PAGE (reduced and nonreduced) with Mr ≈20 kDa, had a single N-terminal sequence by mass spectrometry, and had a lipopolysaccharide content of <10 pg/ml (at a protein concentration of 3-5 mg/ml based on the Endospecy chromogenic assay; Seigaku). The protocol for binding included washing cultured endothelium ($2x10^4$ cells/well) in Hanks' balanced salt solution, and then adding Minimal Essential Medium containing fetal bovine serum (10%) at 4°C containing ^{125}I-rEMAP II alone or in the presence of an 100-fold molar excess of unlabelled rEMAP II. Wells were incubated for 2 hrs at 4°C, unbound material was removed by six rapid washes (for a total of 6 sec/well) with phosphate-buffered saline, and cell-associated radioactivity was eluted with phosphate-buffered saline containing Nonidet

P-40 (1%). Specific binding (total binding observed in wells incubated with ^{125}I-rEMAP II alone minus nonspecific binding observed in wells incubated with ^{125}I-rEMAP II + excess unlabelled rEMAP II) is shown in the figures, and was analyzed by the method of Klotz and Hunston (Klotz, I., 1984).

Matrigel model. Matrigel (Collaborative Research)(Kleinman, H., 1986; Passaniti, A., 1992) containing either vehicle (1% bovine serum albumin), VEGF (100 ng/ml; Collaborative Research) + vehicle, or heat-inactivated VEGF (15 min at 100°C) + vehicle (mouse serum albumin, 1 mg/ml) was mixed at 4°C. Matrigel mixtures (0.25 ml/site; two sites per animal) were injected subcutaneously into C57BL6/J mice (0.25 ml/site) at two sites per animal. Animals were treated with rEMAP II (1 μg/12 hrs; IP) starting on the day that Matrigel was implanted, and for the next 14 days, at which time Matrigel was harvested. Vascular ingrowth was analyzed by routine histology and hemoglobin assay (Sigma). Each experiment employed five animals per group.

Tumor models. All protocols were approved by the Columbia IACUC. C6 glioma cells were implanted into the frontal lobe of Wistar rats (250-300 grams; Charles River) by a modification of methods described in the literature (San-Galli, F., 1989; Bernstein, J., 1990). In brief, following anesthesia with ketamine (90 mg/kg; IP Parke-Davis, NJ) and xylazine (10 mg/kg; IP; American Health Co., Kansas), animals were placed in the sterotactic head frame, a 2-3 mm hole was made in the skull, the dura was opened, and the stereotactic apparatus was used to place a rod (23 gauge stainless needle) 4.5 mm into the deep white matter of the right frontal lobe (coordinates for the burr hole were 1 mm anterior to the coronal suture and 3 mm lateral to the sagittal suture). Rats were allowed to recover for 48 hrs, and then, following anesthesia, C6 glioma cells were administered ($4x10^4$ cells in 5 μl of Hanks balanced salt solution) at an infusion rate of 1 μl/min. After an

additional 10 days, tumor-bearing rats were divided eight different treatment groups (N=9 per group for a total of 72 animals/experiment): (1) rEMAP II (100 ng) administered intratumorally (IT) every 48 hrs (40 μl over 133 min) in vehicle (rat serum albumin, 1 mg/ml) + rEMAP II (10 μg) administered intraperitoneally (IP) every 12 hrs in vehicle; (2) rEMAP II (100 ng) IT in vehicle; (3) rEMAP II (10 ng) IT in vehicle + rEMAP II (1 μg) IP in vehicle; (4) rEMAP II (10 μg) IP in vehicle; (6) vehicle IT every 48 hrs + vehicle IP every 12 hrs; (7) vehicle IP every 12 hrs; (8) vehicle IT every 48 hrs; and, (9) heat-inactivated rEMAP II (100 ng) IT in vehicle every 48 hrs + heat-inactivated rEMAP II (10 μg) IP in vehicle every 12 hrs. Intratumoral administration involved positive pressure microinfusion through the implanted rod at a volume of 40 μl infused over 133 min. Once the treatment regimen including rEMAP II was begun, it was continued for a total of either 7 or 14 days. There were 8 eight animals in each treatment group. At the end of the experiment, animals were sacrificed by humane euthanasia, the cranium was opened, the brain removed, incubated in formalin (4%) at 4°C for 72 hrs, and placed in a brain matrix to make serial 1 mm coronal slices. The latter were paraffin-embedded, sectioned coronally (4 μm), and underwent routine histology (hematoxylin/eosin) and TUNEL assay (see below). Tumor volume was calculated according to the formula for a spherical segment (see below; Weast R., 1966) based on the largest cross-sectional tumor diameter, and serial images were evaluated by NIH image.

Tumors were grown subcutaneously in nude mice (Taconic) by administering C6 glioma cells ($2x10^6$ suspended in phosphate-buffered saline; Kim, K., 1993; Millauer, B., 1994), and on the third day animals were divided into thre groups (N=10-15/group): (1) vehicle (mouse serum albumin, 1%) given IP every 12 hrs; (2) rEMAP II (1 μg) in vehicle given IP every 12 hrs; and, Folkman, J., 1995) heat-inactivated rEMAP II (1 μg) in vehicle given IP every 12 hrs. Initial tumor size (i.e., prior to treatment on day

3) in each of the groups was 12-14 mm^3. This treatment regimen was continued for up to 31 days. Tumor dimensions were measured every fourth day, and these data were used to calculate tumor volume according to the formula for a spherical segment (Weast R., 1966), $V= \pi h(h^2+3a^2)/6$, where h= height of the segment, a= (length+width)/2, and V= volume (each tumor was compared with itself over multiple measurements and change in volume was noted). Tumor volume data were analyzed using the Kruskal-Wallis one way ANOVA and a Mann-Whitney mean rank test. Data is expressed as a dimensionless ratio of observed tumor volume divided by initial (day 3) tumor volume. Animals were sacrificed and tumors analyzed histologically at the indicated times.

Histologic studies were performed on formalin-fixed, paraffin-embedded tissue, using hematoxylin and eosin staining. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) assay was used to evaluate apoptosis; paraffn embedded tumor slices were deparaffinized and digoxigenin-11-dUTP was used to label fragmented DNA according to the Genius 1 kit (Amersham). In brief, tissue was treated with proteinase K (1 μg/ml), and incubated with digoxigenin-11-dUTP, klenow, and dNTPs overnight. Nitroblue tetrazolium and alkaline phosphatase were used to reveal the digoxigenin labelled DNA fragments.

Ultrastructural studies. Glioblastomas were raised subcutaneously in nude mice, and tumors were removed and processed for electron microscopy on days 3, 6 and 9 (Roberts, W., 1995). In brief, tumor specimens were cut into small pieces and immediately fixed by immersion in glutaraldehyde (1.5%) in sodium cacodylate-HCl (0.1 M; pH 7.4) with sucrose (5%) for 1 hr. Following washes in cacodylate buffer (0.1 M) with sucorse (7.5%), specimens were post-fixed in cacodylate-buffered (pH 7.4) osmium tetroxide (1%) for 1 hr, en-bloc staine with uranyl acetate overnight, dehydrated, embedded in EPON 812, and cured for

18-24 hrs at 60°C. Thin (50-55 nm) sctions were cut (Reichert-Jung Ultracut E, Austria) piked up on copper grids, stained with uranyl acetate and lead citrate before examination and photographing on a Phillips CM10 electron microscope at 80 kV.

RESULTS

Effect of rEMAP II on growth of C6 gliomas in vivo. C6 glioma cells were implanted stereotactically in the right frontal lobe of Wistar rats. This model was selected based on previous studies demonstrating that histologic features of these tumors closely resemble findings in tumors of patients (San-Galli, F., 1989; Bernstein, J., 1990). Tumor growth occurred steadily up to about 28 days, when death resulted from increased intracranial pressure. For this reason, experiments were terminated at day 24; there were no fatalities at this time. To assess the effect of rEMAP II on growth of C6 gliomas, three different treatment regimens were employed: intra-tumoral (IT) administration via indwelling cannula, intraperitoneal (IP) administration, and both IT + IP (IT/IP) administration. IT treatment, via indwelling cannula according to our protocol, has been shown to effectively deliver therapeutic agents within the central nervous system without elevating intracranial pressure. Animals receiving rEMAP II by the IT/IP routes showed the greatest suppression of tumor growth; at a dose of 100 ng (IT)/10 μg (IP), tumor volume was diminished by ≈3-fold compared with tumors treated according to the same protocol with vehicle alone (IT/IP, IP or IT) ($P \leq 0.002$). The effect of rEMAP II was dose-dependent, being diminished at 10 ng (IT)/1 μg (IP), in which case tumor volume was diminished, but did not achieve statistical significance compared with controls. Rats treated with rEMAP II (10 μg) by the IP route alone showed little change in tumor growth, whereas rEMAP II (100 ng) given only IT had a striking effect, though it was somewhat less effective than combined IP/IT administration. Controls in which heat-inactivated rEMAP II replaced active rEMAP II demonstrated no reduction in tumor

size compared with vehicle-treated controls. Histologic analysis of control tumors (either no treatment or IP/IT vehicle alone) showed a relatively homogeneous central region of the tumor (Fig. 9B-9C) with areas of palisading tumor cells and necrosis at the periphery. In the central region of the tumor from control animals, where necrosis was minimal, there was also little evidence of DNA fragmentation, based on the TUNEL assay (Fig.9D-9E). In contrast, tumors from animals receiving rEMAP II (IP/IT) demonstrated marked inhomogeneity with pyknotic areas (Fig. 9C-9D) in which DNA fragmentation was ubiquitous. Quantitative analysis using NIH image indicated that C6 gliomas treated with rEMAP II (IP/IT) had ~80% of the residual volume accounted for by apoptotic cells. As the treatment regimen with rEMAP II was modified from 100 ng (IP)/10 μg (IT) to lower concentrations, either 10 ng (IT)/1 μg (IP), or 1 μg (IP) or 100 ng (IT) alone, resulting in less effective suppression of tumor growth, histologic evidence of pyknosis and apoptosis decreased. In contrast to the effect of rEMAP II (IP/IT) on tumors, there was no evidence of toxicity even after 14 days of treatment. There were no deaths in the treatment group; animals displayed normal activity, behavior (no seizures or other untoward events were observed), and food intake.

To further examine the effect of rEMAP II on C6 gliomas, experiments were performed with tumor cells inoculated subcutaneously in nude mice. Tumor cells were implanted into immunocompromised mice, and growth was allowed to occur for three days, at which time palpable tumors were reproducibly evident (approximate volume prior to treatment was 12-14 mm^3, in each group). rEMAP II was then administered starting on day 3, and tumor volume was measured every four days thereafter; data are reported at each time point as fold-change in tumor volume (a dimensionless ratio comparing tumor volume on the indicated day with that on day 3); this method allowed a comparison of each animal with itself. Tumors in rEMAP II-treated animals

displayed ~6-fold reduction in volume compared with tumors in mice receiving vehicle alone by day 31 (Fig. 9E). Histologic appearance of rEMAP II-treated C6 gliomas showed small tumors with evidence of pyknotic changes and apoptosis throughout the lesions, compared with larger tumors in vehicle-treated controls which displayed homogeneous central areas and apoptotic/necrotic changes limited to the periphery.

VEGF-mediated vascular ingrowth into Matrigel implants: effect of rEMAP II. Matrigel is a complex mixture of basement membrane proteins, as well as other cell products, from Engelbreth-Holm-Swarm (EHS) tumor cells (Kleinman, H., 1986; Passaniti, A., 1992). Addition of an exogenous growth factor, such as basic fibroblast growth factor, to Matrigel has been shown to provide a model for assessment of vessel ingrowth (Kleinman, H., 1986; Passaniti, A., 1992). This model was employed by mixing Matrigel with recombinant human VEGF and subcutaneously implanting the mixture into mice. Animals were then treated with either rEMAP II (1 μg) in vehicle, heat-inactivated rEMAP II (1 μg) in vehicle or vehicle alone every 12 hrs for 14 days. Implants containing VEGF in animals not receiving active rEMAP II (i.e., vehicle controls or heat-inactivated rEMAP II) showed numerous vascular structures (Figs. 10A-B show the results with vehicle alone administered IP and VEGF in the Matrigel implant). Vascular ingrowth was markedly inhibited in the presence of active rEMAP II administered IP (Fig. 10C-D). Consistent with these results, hemoglobin assays on Matrigel implants, as an estimate of vascularization, showed reduced hemoglobin content in samples from animals treated with rEMAP II (Fig. 10E). Controls in which VEGF was heat-inactivated demonstrated suppression of the angiogenic response, whereas heat-inactivation of rEMAP II in implants containing active VEGF did not diminish neovessel formation. These data suggested that the vasculature was a target of EMAP II, and led us to re-assess normal organs in animals treated with rEMAP II. No change was observed in micro- or

macro-vessels from the organs, such as heart, lung, spleen, or kidney), suggesting that the effects of EMAP II were focussed on the tumor bed.

Ultrastructural properties of tumor vasculature: effect of rEMAP II. Tumors harvested after 3, 6 or 9 days of EMAP II treatment were noticeably different from controls. Macroscopically, reddish pinpoint areas were observed, presumably the result of red blood cell stasis, extravsation and thrombus formation. This impression was confirmed by microscopic studies showing platelet thrombi and red cell stasis, especiallly in large (40 μm dimabeter) venular vessels. Consistent with the presence of fibrin, ultrastructural studies showed a 21 nm periodicity of the fibrin strands. Vasculature in both control and EMAP II-treated tumors displayed attentuated endothelium, often with fenestrations and open interendothelial junctions (Roberts, W., 1995). However, platelet thrombi and fibrin clots were noticed only in EMAP II-treated tumors.

Interaction of rEMAP II with cultured endothelial cells. These data suggested that EMAP II selectively interacted with vascular elements in the tumor bed. In support of this concept, human umbilical vein endothelial cells exposed to rEMAP II showed increased DNA fragmentation by agarose gel electrophoresis, compared with untreated controls (Fig. 11A). Also, ^{125}I-rEMAP II bound to cultured endothelium in a dose-dependent manner, demonstrating K_d = 1.9 nM (Fig. 11B). In contrast to these results with endothelial cells, rEMAP II had no affect on C6 glioma cells with respect to apoptosis (or changes in cell number). Also, there was no specific binding of ^{125}I-rEMAP II to cultured C6 glioma cells. These data suggest the existence of functional rEMAP II binding sites/receptors on endothelium.

DISCUSSION

Recent studies on glioblastoma multiforme have focussed attention on tumor vasculature as a model system for

analysis of angiogenic mechanisms and for examination of potential future therapeutic modalities (San-Galli, F., 1989; Plate, K., 1992; Wesseling, P., 1994; Plate, K., 1995; Shweiki, D., 1992; Weindel, K., 1994; Plate, K., 1994; Samoto, K., 1995; Kim, K., 1993; Millauer, B., 1994; Saleh, M., 1996). Areas of necrosis at the tumor margin, along with the presence of palisading tumor cells which express VEGF, have emphasized the possibility that induction of vascular ingrowth is a limiting factor for growth of the neoplasm, and that VEGF may have a central role in tumor survival. This concept is supported by the results of studies demonstrating that antagonism of VEGF with specific antibodies, at the level of the endothelial receptor Flk-1, or with antisense technology supresses tumor formation (Kim, K., 1993; Millauer, B., 1994; Saleh, M., 1996). The current data add to and extend the concept that a mediator with properties which impact negatively on vascular integrity might inhibit growth of glioblastomas. Further support for the value of such an approach to therapy of glioblastomas is also provided by recent studies outlining anti-angiogenic strategies for glioma therapy (Johnson, J., 1996).

EMAP II has several properties which are consistent with the hypothesis that it specifically affects tumor vasculature. First, experiments in tissue culture demonstrate specific, high affinity binding to endothelium, but not to C6 glioma cells. Second, EMAP II induction of endothelial apoptosis appears limited to growing endothelium, a situation particularly relevant to tumor vasculature (Fidler, I., 1994; Folkman, J., 1989; Folkman, J., 1995; O'Reilly, M., 1994; Holmgren, L., 1995). These observations in tissue culture provide support for the results of our in vivo studies in which animals receiving EMAP II demonstrated suppression of tumor growth, but did not display toxic effects in the vascular bed of normal organs. Third, EMAP II administered systemically antagonizes VEGF-induced neovessel formation in the Matrigl model, whereas no untoward reactions occurred in established vasculature

outside the implant. In addition to antagonism of VEGF, EMAP II may provide a broader spectrum of activities which impact negatively on tumor survival in the host, including inhibition of other angiogenic activities in the tumor, such as basic fibroblast growth factor (Stan, A., 1995). Further, EMAP II induction of endothelial tissue factor (Kao, J., 1992; Kao, J., 1994) potentially underlies vascular fibrin formation, ultimately leading to occlusive thrombosis and cessation of blood flow. Ultrastructural analysis of tumor vasculature from EMAP II-treated animals confirmed the presence of both fibrin and findings consistent with apoptosis of the endothelium.

Although it is difficult to dissect with certainty the exact mechanism through which EMAP II exerts its affects on tumors from the pathologic picture in treated tumors bed of tumors treated with EMAP II, a mechanism other than direct tumor cell cytotoxicity seems likely. If this proves to be true, the most effective therapy might be to combine EMAP II with agents directly targetting neoplastic cells, such as cytotoxic agents or anti-sense to Insulin-like Growth Factor, the latter having been shown to suppress glioma growth (Resnicoff, M., 1994). One possible insight into the complexity of EMAP II's actions in the tumor bed is suggested by the greatly enhanced anti-tumor effect following intratumor injection. The observation herein that EMAP II administered only via the systemic route (IP) had little effect on tumor growth suggests that adequate access to the central nervous system was not possible following systemic delivery. Delivery of anti-tumoral compounds to brain tumors is compromised by systemic methods which limit effective drug concentrations due to limitations imposed by the blood brain barrier (as with EMAP II which is a polypeptide of 22 kDa) and/or systemic degradation/clearance of compounds (Tomita, T., 1991). These limitations can be safely circumvented by local delivery methods utilizing high flow positive pressure microinfusion directly into the tumor through the interstitial spaces by bulk flow. The drug

concentration drops off rapidly at the edge of the infusion front (i.e., high local concentrations of drug can be achieved) and adjustments can be made in the rate and volume of the infusion to achieve desired constant tissue concentrations. Bulk flow is less dependent on the physical characteristics of the substance being infused and is therefore uniquely suitable for complex macromolecues which do not diffuse well and are difficult to deliver systemically. Efficacy of this delivery system, without complications, using EMAP II demonstrates the potential for clinical development of other novel compound for anti-tumoral therapeutics that would otherwise be impractical if delivered systemically.

Delivering therapeutic agents to the central nervous system poses special difficulties because of the presence of the blood-brain barrier. Although neovasculature in glioblastomas displays histologic features which suggest increased permeability, the likelihood of gross breakdown of the blood-brain barrier is low. The ability to deliver EMAP II via an indwelling catheter in the region of the tumor, whithout raising intracranial pressure or causing other untoward effects in the central nervous system, introduces the therapeutic agent in proximity to glioma cells, the primary source of angiogenic stimuli. Combined therapy, both systemic and local, was most efficacious, possibly by maximizing delivery of EMAP II to both tumor vessels, interstitium and the neoplastic cells. These data emphasize a role for local delivery of therapeutic agents to intracranial tumors, and suggest the importance of developing and testing such systems which can be ultimately applied to clinical practice.

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What is claimed is:

1. A method of treating a tumor in a subject, comprising administering to the subject an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to treat the tumor, wherein the endothelial monocyte activating polypeptide II is administered subcutaneously, intraperitoneally, or intravenously.

2. The method of claim 1, wherein the tumor is a carcinoma.

3. The method of claim 1, wherein the subject is a mouse.

4. The method of claim 1, wherein the agent is administered intraperitoneally.

5. The method of claim 1, wherein the agent is administered in at least twenty doses.

6. The method of claim 5, wherein the agent is administered in about twenty-four doses.

7. The method of claim 1, wherein the agent is administered over a period of at least ten days.

8. The method of claim 7, wherein the agent is administered over a period of about twelve days.

9. The method of claim 1, wherein the frequency of administration is at least about one dose every twelve hours.

10. The method of claim 1, wherein the effective amount is from about 2.4 micrograms to about 24 micrograms.

11. The method of claim 1, wherein the effective amount is from about 100 nanograms to 24 micrograms per dose.

12. The method of claim 11, wherein the effective amount is from about 100 nanograms to about 1000 nanograms per dose.

13. The method of claim 1, wherein the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence

(S/M/G)KPIDASRLDLRIG
(C/R)IVTAKKHPDADSLYVEEVDVGEAAPRTVVSGLVNHVPLEQMQNRMVVL
LCNLKPAKMRGVLSQAMVMCASSPEKVEILAPPNGSVPGDRITFDAFPGEPDK
ELNPKKKIWEQIQPDLHTNAECVATYKGAPFEVKGKGVCRAQTMANSGIK,

wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues.

14. The method of claim 13, wherein the homology is at least about ninety-five percent.

15. The method of claim 1, wherein the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence

(S/M/G)KPIDVSRLDLRIG
(C/R)LITARKHPDADSLYVEEVDVGEIAPRTVVSGLVNHVPLEQMQNRMVIL
LCNLKPAKMRGVLSQAMVMCASSPEKIEILAPPNGSVPGDRITFDAFPGEPDK
ELNPKKKIWEQIQPDLHTNDECVATYKGVPFEVKGKGVCRAQTMSNSGIK,

wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues.

16. The method of claim 15, wherein the homology is at least about ninety-five percent.

17. The method of claim 1, wherein the agent is endothelial monocyte activating polypeptide II.

18. The method of claim 17, wherein the endothelial monocyte activating polypeptide II is murine endothelial monocyte activating polypeptide II.

19. The method of claim 17, wherein the endothelial monocyte activating polypeptide II is human endothelial monocyte activating polypeptide II.

20. The method of claim 17, wherein the endothelial monocyte activating polypeptide II is recombinant endothelial monocyte activating polypeptide II.

21. The method of claim 1, wherein the tumor is too small for intratumoral injection.

22. The method of claim 21, wherein the diameter of the tumor is less than or equal to about two millimeters.

23. A method of inhibiting the growth of endothelial cells, comprising contacting the endothelial cells with an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to inhibit growth of the endothelial cells.

24. The method of claim 23, wherein the endothelial cells are aortic endothelial cells.

25. A method of inhibiting the formation of blood vessels in a subject, comprising administering to the subject an effective amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, thereby inhibiting the formation of blood vessels in the subject.

26. The method of claim 25, wherein the subject is a mouse.

27. The method of claim 25, wherein the agent is administered subcutaneously, intravascularly, intraperitoneally, topically, or intramuscularly.

28. The method of claim 25, wherein the effective amount is from about 10 nanograms to about 24 micrograms.

29. The method of claim 28, wherein the effective amount is from about 100 nanograms to about 1 microgram.

30. The method of claim 25, wherein the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDASRLDLRIG (C/R)IVTAKKHPDADSLYVEEVDVGEAAPRTVVSGLVNHVPLEQMQNRMVVL LCNLKPAKMRGVLSQAMVMCASSPEKVEILAPPNGSVPGDRITFDAFPGEPDK ELNPKKKIWEQIQPDLHTNAECVATYKGAPFEVKGKGVCRAQTMANSGIK, wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues.

31. The method of claim 30, wherein the homology is about ninety-five percent.

32. The method of claim 25, wherein the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDVSRLDLRIG (C/R)IITARKHPDADSLYVEEVDVGEIAPRTVVSGLVNHVPLEQMQNRMVIL LCNLKPAKMRGVLSQAMVMCASSPEKIEILAPPNGSVPGDRITFDAFPGEPDK ELNPKKKIWEQIQPDLHTNDECVATYKGVPFEVKGKGVCRAQTMSNSGIK, wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues.

33. The method of claim 32, wherein the homology is at least about ninety-five percent.

34. The method of claim 25, wherein the agent is endothelial monocyte activating polypeptide II.

35. The method of claim 25, wherein the endothelial monocyte activating polypeptide II is murine endothelial monocyte activating polypeptide II.

36. The method of claim 25, wherein the endothelial monocyte activating polypeptide II is human endothelial monocyte activating polypeptide II.

37. The method of claim 25, wherein the endothelial monocyte activating polypeptide II is recombinant endothelial monocyte activating polypeptide II.

38. A method of treating a condition involving the presence of excess blood vessels in a subject, comprising the method of claim 25.

39. The method of claim 25, wherein the condition involves the presence of excess blood vessels in the eye.

40. The method of claim 39, wherein the condition is a retinopathy.

41. The method of claim 40, wherein the retinopathy is diabetic retinopathy.

42. The method of claim 40, wherein the retinopathy is sickle cell retinopathy.

43. The method of claim 40, wherein the retinopathy is retinopathy of prematurity.

44. The method of claim 40, wherein the retinopathy is age related macular degeneration.

45. A method of treating a tumor in a subject, comprising

administering to the subject an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to treat the tumor, wherein the endothelial monocyte activating polypeptide II is administered subcutaneously or intraperitoneally; and intravenously, intracranially, or intramorally.

46. The method of claim 45, wherein the tumor is a glioblastoma.

47. The method of claim 45, wherein the agent is administered intratumorally by positive pressure microinfusion.

48. The method of claim 45, wherein the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDASRLDLRIG (C/R)IVTAKKHPDADSLYVEEVDVGEAAPRTVVSGLVNHVPLEQMQNRMVVL LCNLKPAKMRGVLSQAMVMCASSPEKVEILAPPNGSVPGDRITFDAFPGEPDK ELNPKKKIWEQIQPDLHTNAECVATYKGAPFEVKGKGVCRAQTMANSGIK, wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues.

49. A method for evaluating the ability of an agent to inhibit growth of endothelial cells, which comprises:

(a) contacting the endothelial cells with an amount of the agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide;

(b) determining the growth of the endothelial cells, and

(c) comparing the amount of growth of the endothelial cells determined in step (b) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit growth of endothelial cells.

50. A method for evaluating the ability of an agent to inhibit the formation of a blood vessel in a cellular environment, which comprises:

(a) contacting the cellular environment with an amount of the agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide;

(b) determining whether or not a blood vessel has formed in the cellular environment, and

(c) comparing the amount of blood vessel growth determined in step (b) with the amount determined in the absence of the agent in the cellular environment, thus evaluating the ability of the agent to inhibit blood vessel formation in the cellular environment.

51. A pharmaceutical composition which comprises an agent capable of inhibiting blood vessel formation and a pharmaceutically acceptable carrier.

52. The pharmaceutical composition of claim 51, wherein the carrier is a diluent, an aerosol, a topical carrier, an aquous solution, a nonaqueous solution or a solid carrier.

FIG. 1

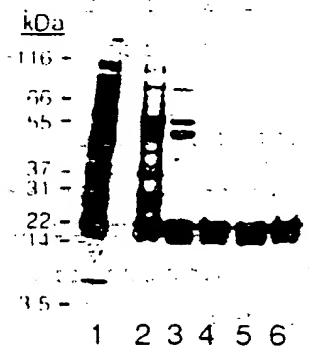
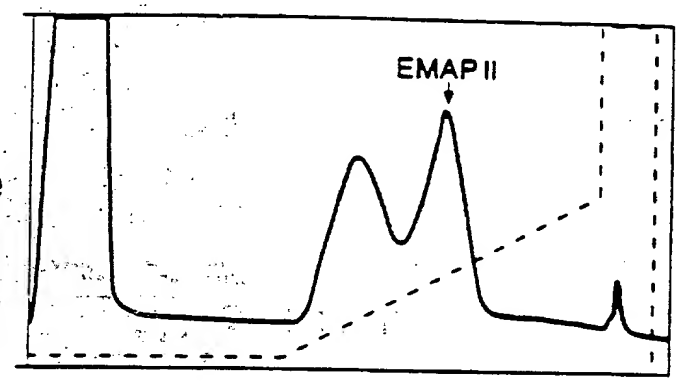
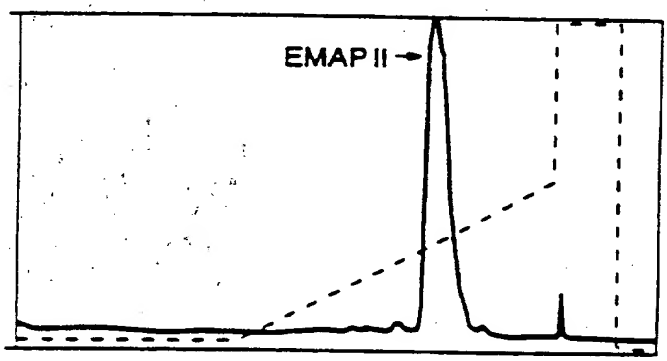


FIGURE 2A



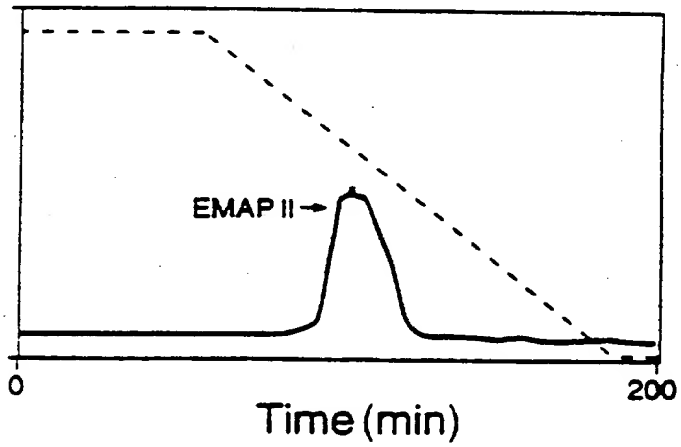
Heparin Sepharose

FIGURE 2B



SP Sepharose

FIGURE 2C



Phenyl Toyopearl

FIG. 3A

FIG. 3B

FIG. 3C

FIG. 3D

FIG. 3E

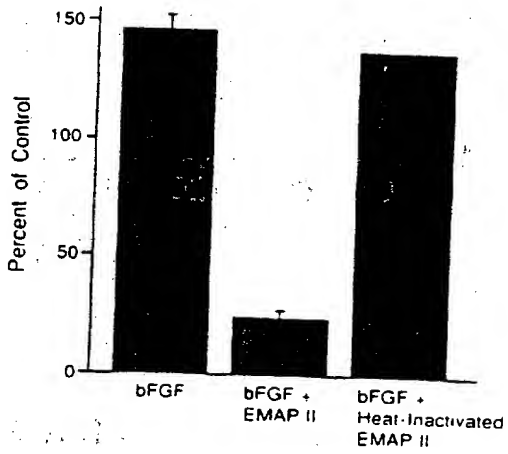

150
100
50
0
Percent of Control
bFGF
bFGF + EMAP II
bFGF + Heat-Inactivated EMAP II

FIGURE 4A

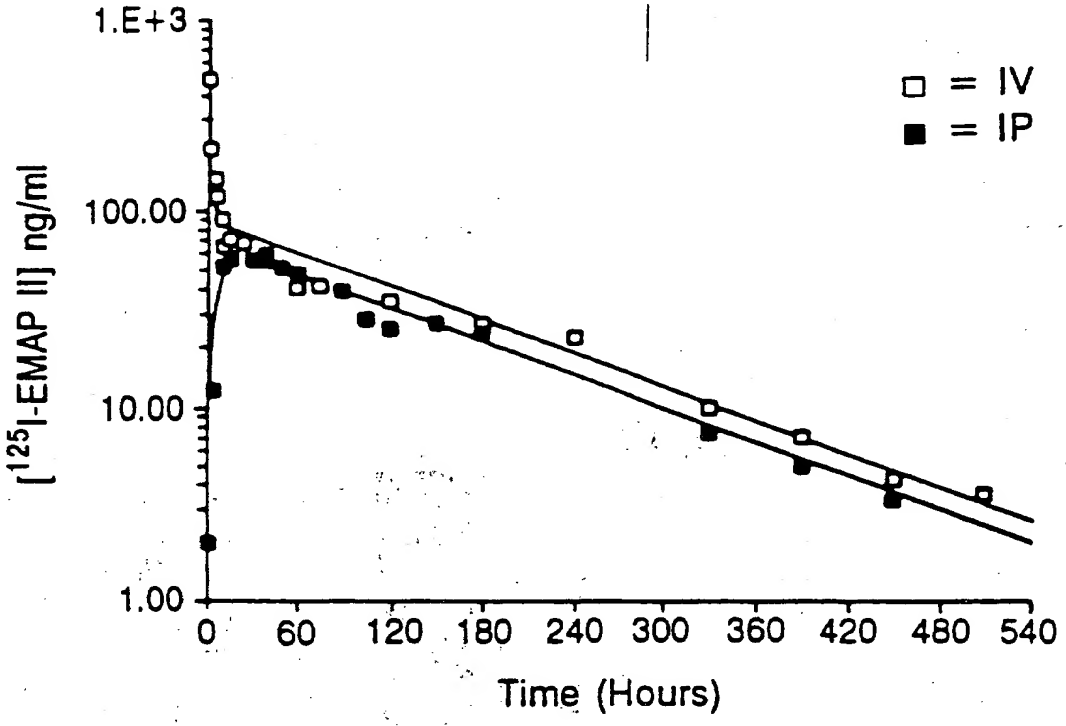


FIGURE 4B

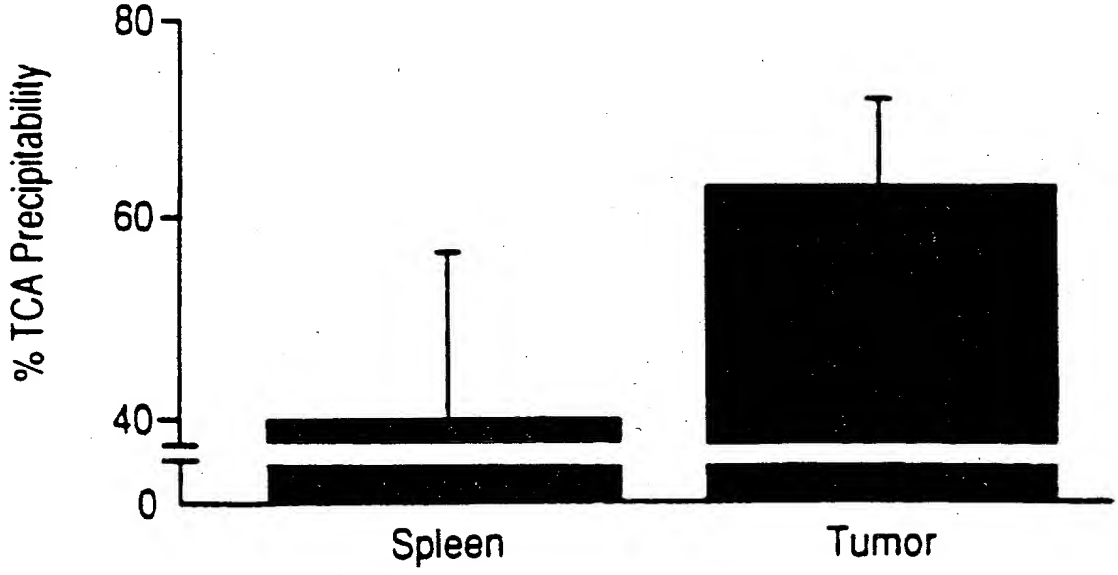


FIGURE 4C

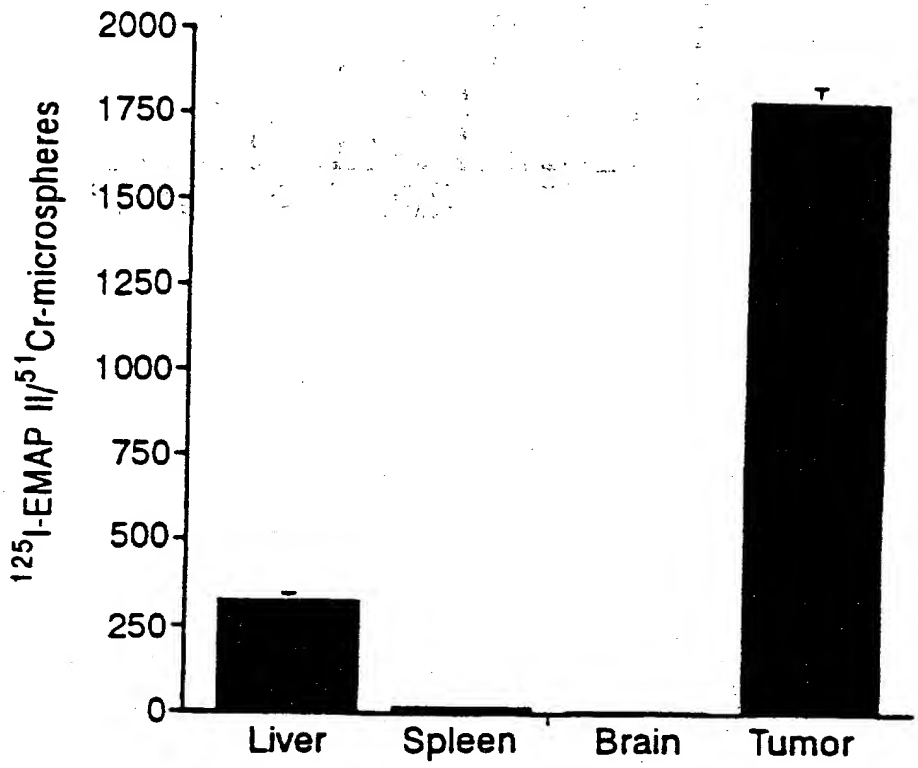

2000
1750
1500
1250
1000
750
500
250
0
^{125}I-EMAP II/^{51}Cr-microspheres
Liver
Spleen
Brain
Tumor

FIG. 5A

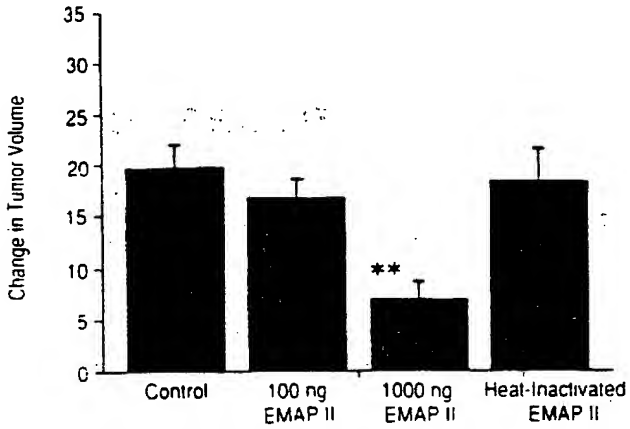

Change in Tumor Volume
35
30
25
20
15
10
5
0
**
Control
100 ng EMAP II
1000 ng EMAP II
Heat-Inactivated EMAP II

FIG. 5B

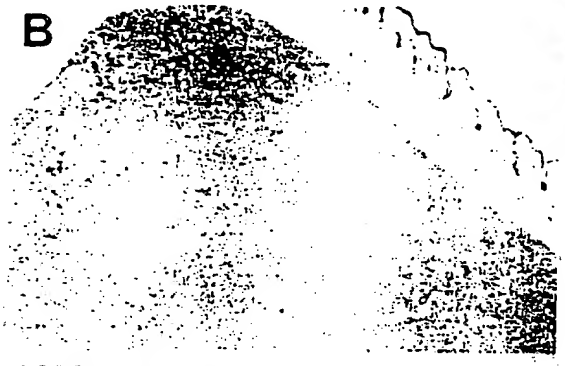


FIG. 5C

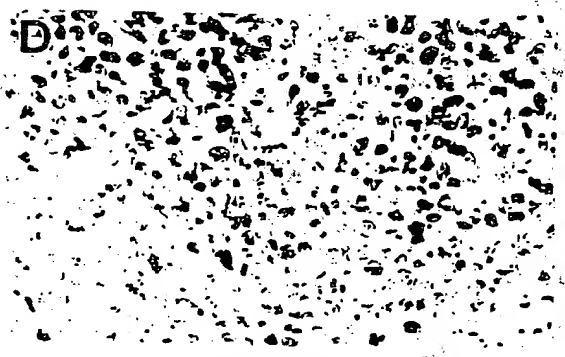


FIG. 5D

FIG. 5E

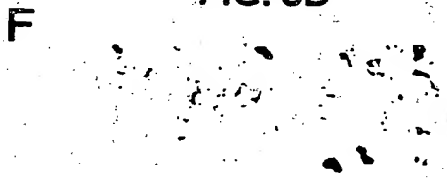


FIG. 5F

FIG. 5G

FIG. 6A

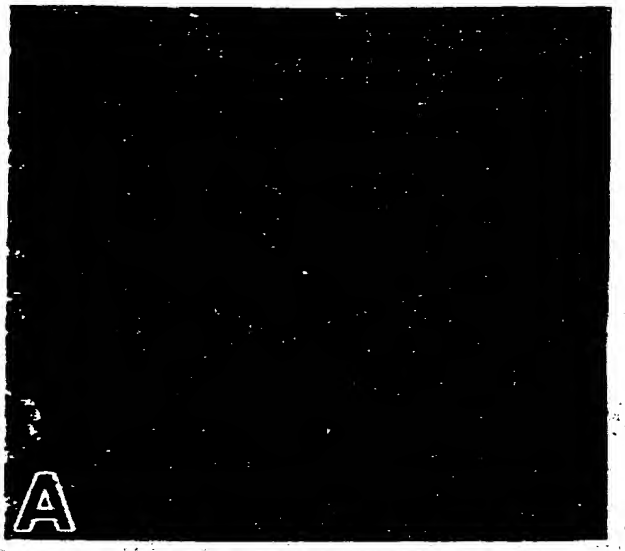
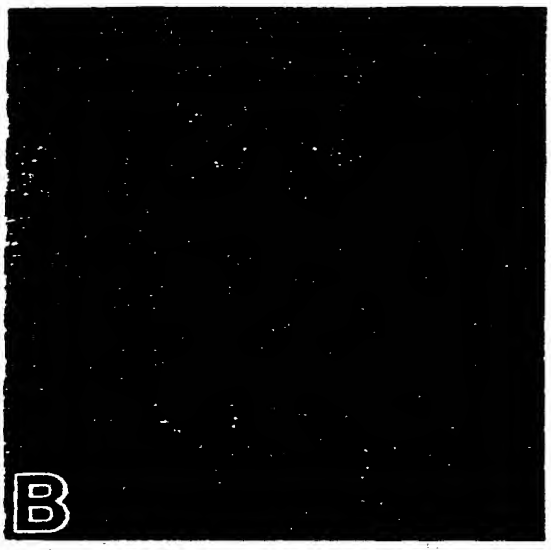


FIG. 6B



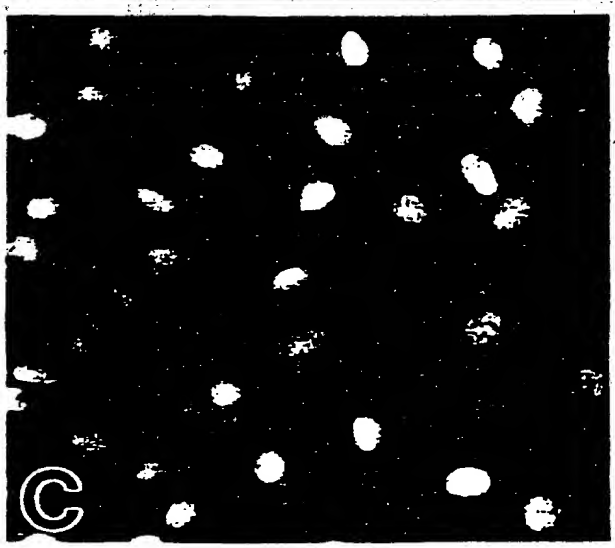


FIG. 6C

FIG. 6D

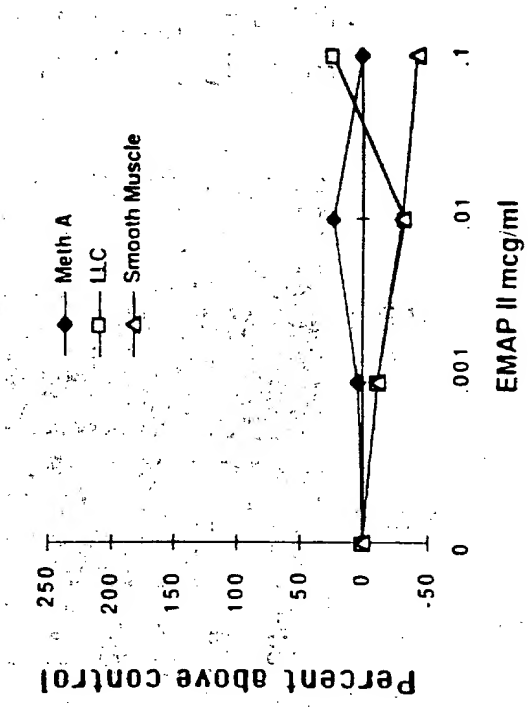

Meth A
LLC
Smooth Muscle
Percent above control
250
200
150
100
50
0
-50
0
.001
.01
.1
EMAP II mcg/ml

FIG. 6F

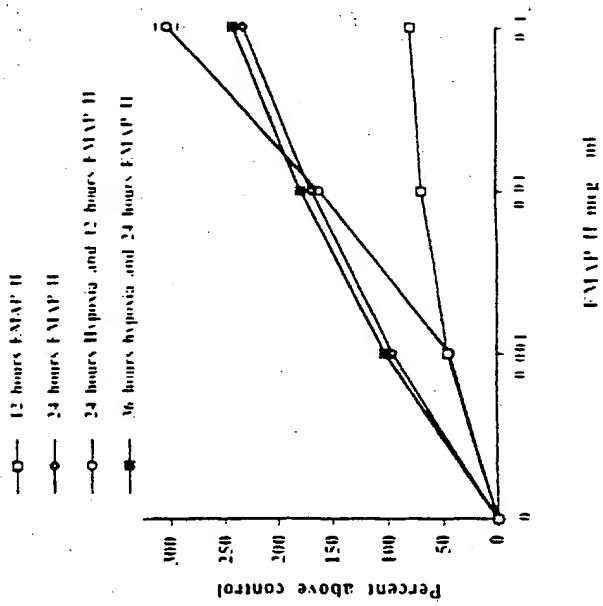

12 hours EMAP II
24 hours EMAP II
24 hours Hypoxia and 12 hours EMAP II
36 hours hypoxia and 24 hours EMAP II
Percent above control
300
250
200
150
100
50
0

FIG. 6E

FIG. 7

PCR Amplification of EMAP II Transcripts in Murine Tissue

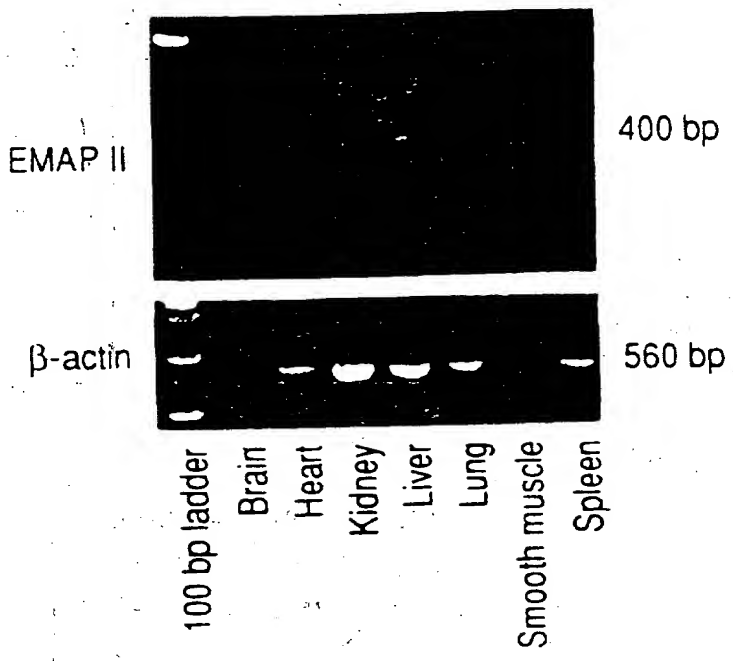


FIG. 8A

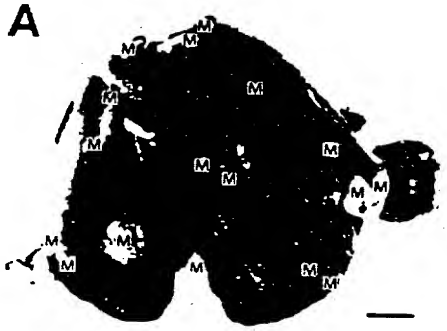
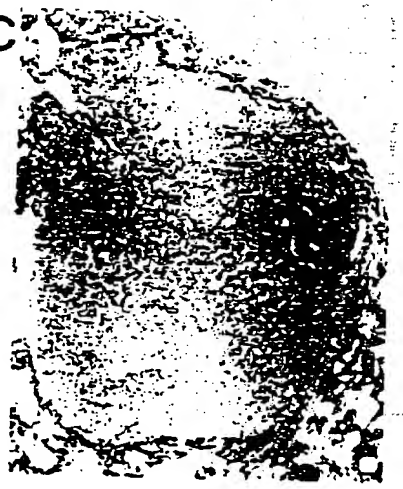


FIG. 8B

FIG. 8C



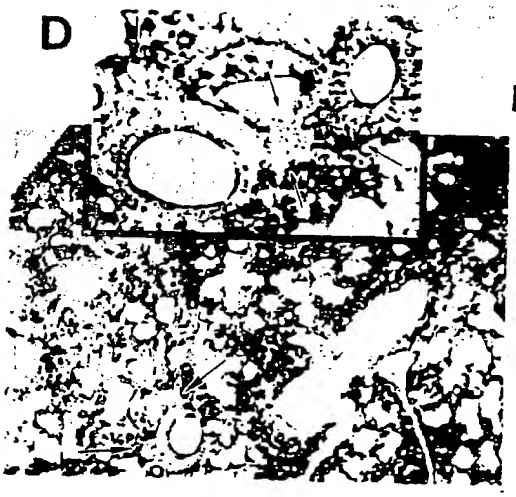


FIG. 8D

FIG. 8E

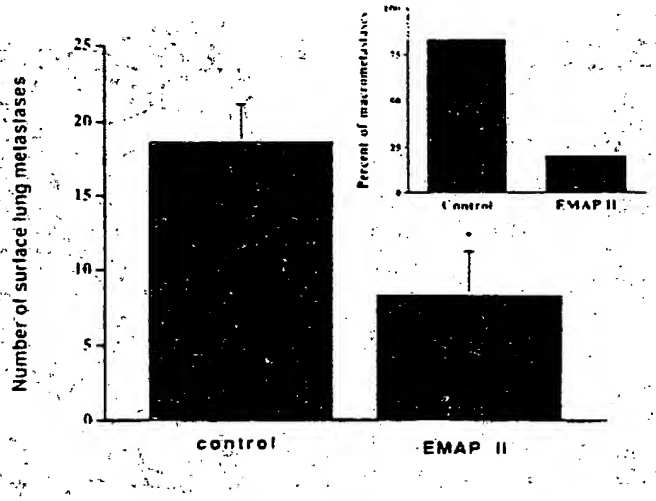
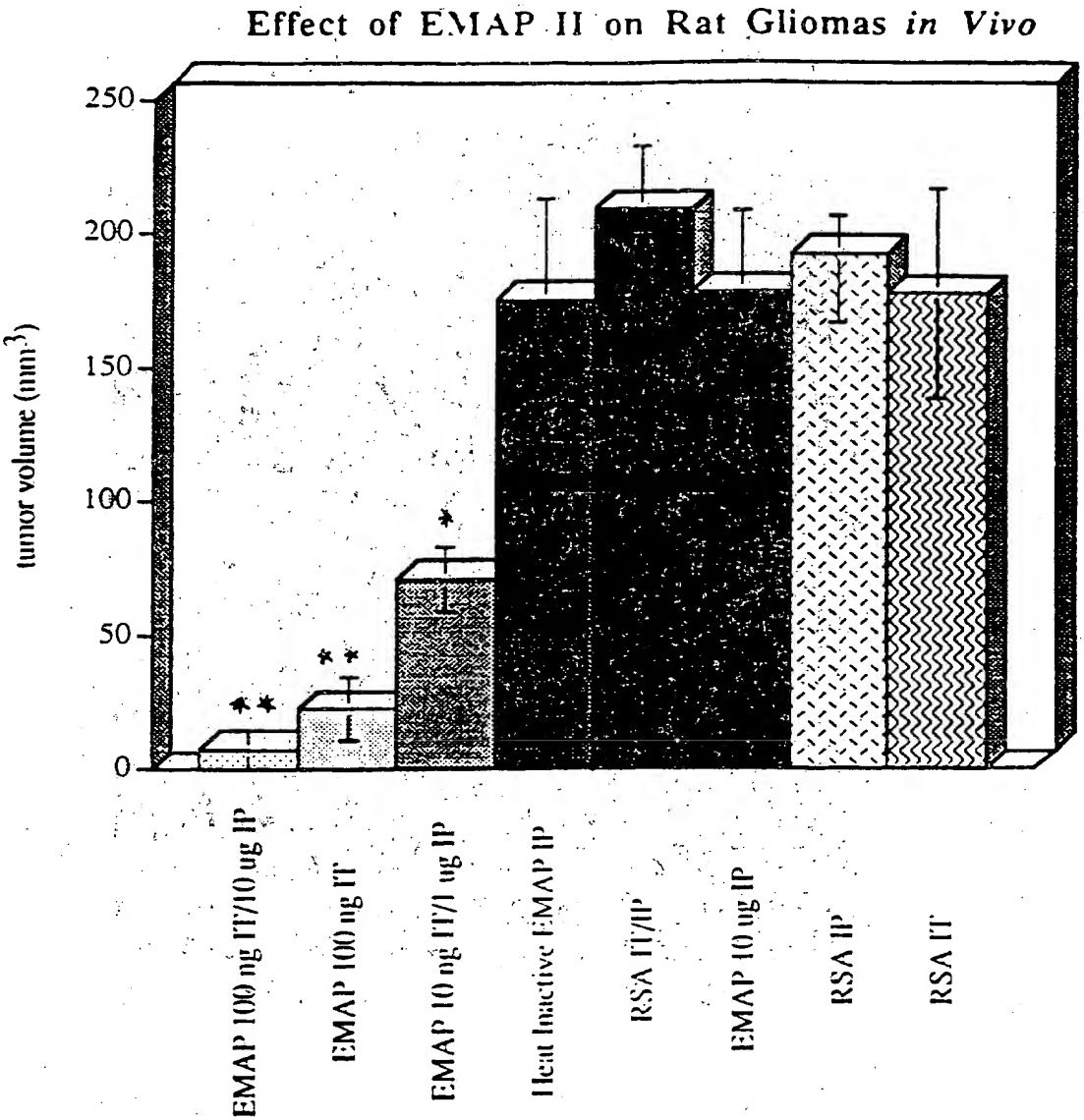

Number of surface lung metastases
25
20
15
10
5
0
control
EMAP II
*
Percent of macrometastases
Control
EMAP II

FIG. 9A



(IT=intratumoral;IP=intraperitoneal;RSA=rat serum albumin)

FIG. 9B

FIG. 9C

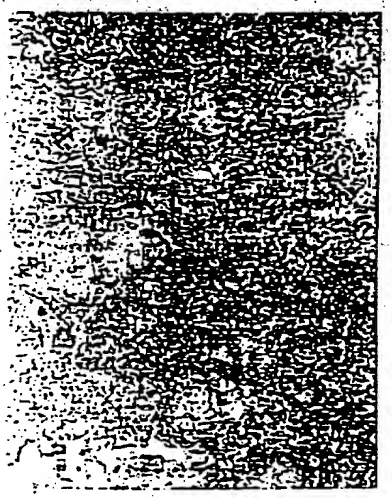


FIG. 9D

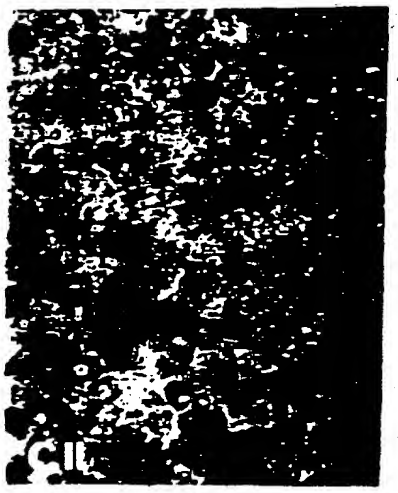


FIG. 9E

FIG. 9F

EMAP II Inhibits Growth of C 6 Gliomas in Immunocompromised Mice.

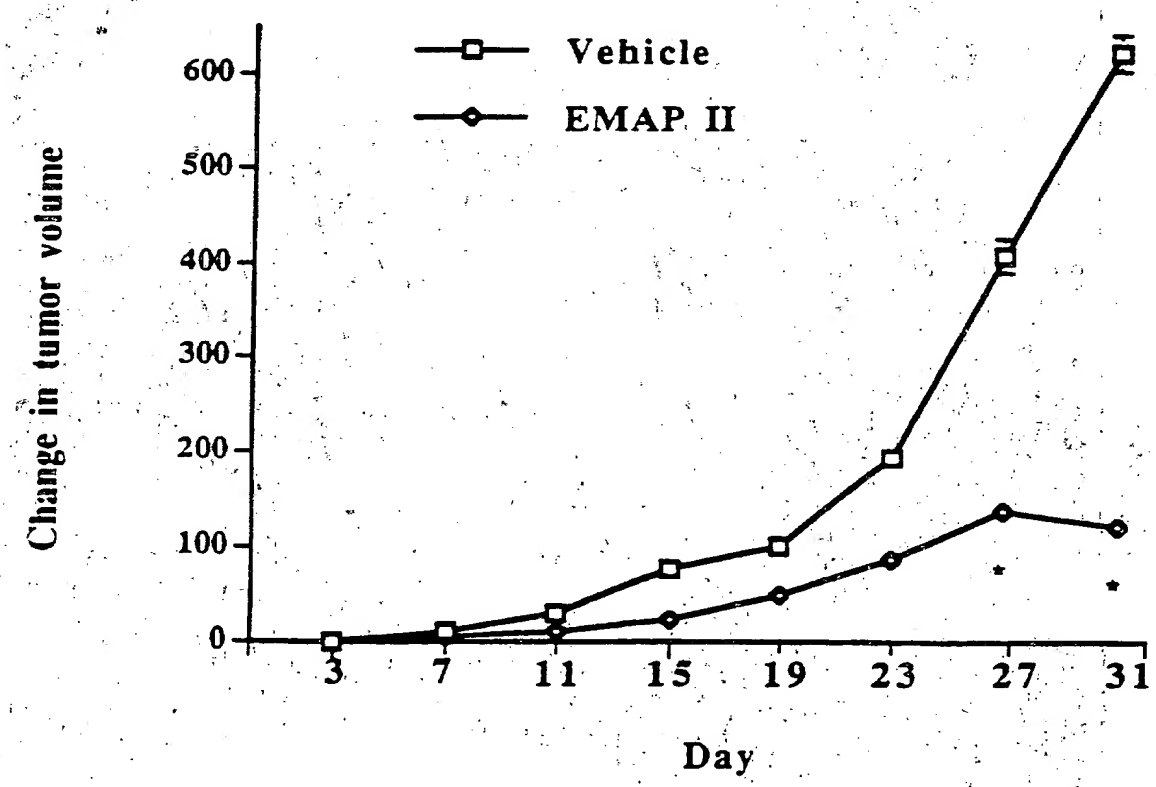


FIG. 10B

FIG. 10A

FIG. 10D

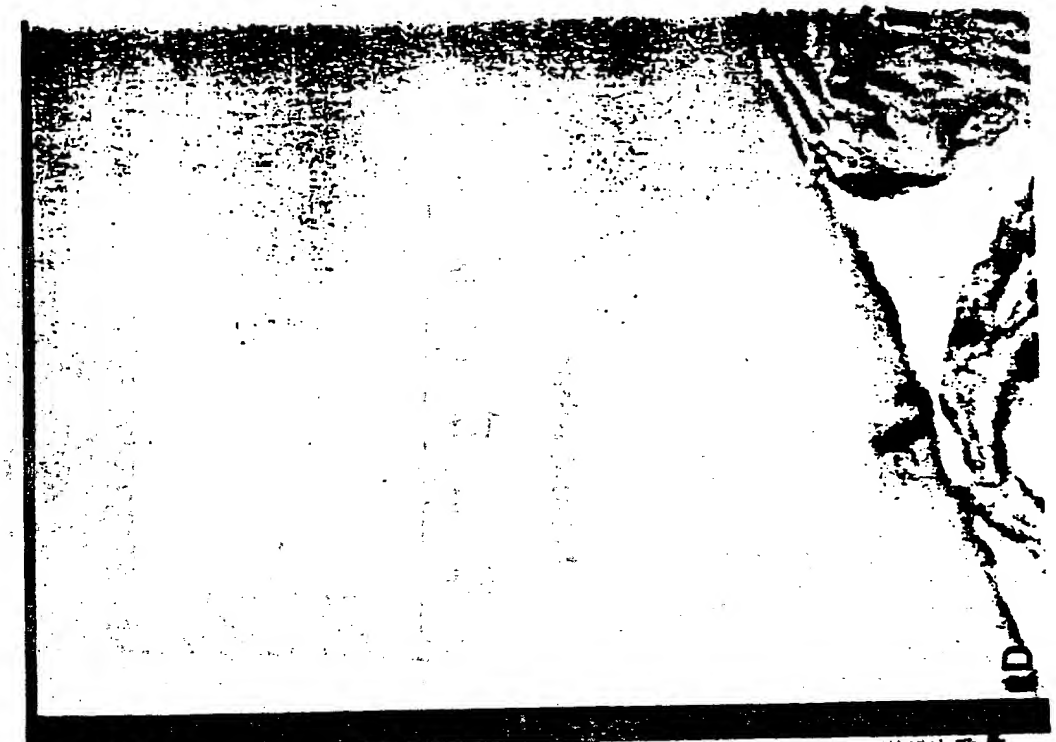


FIG. 10C

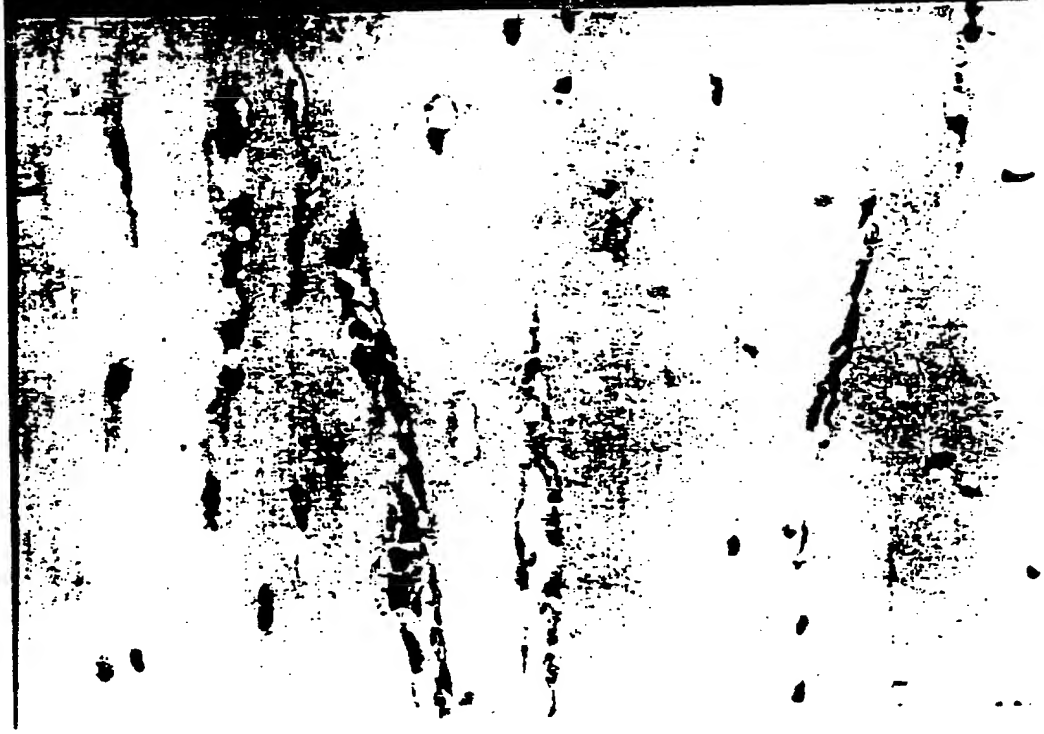


FIG. 10E

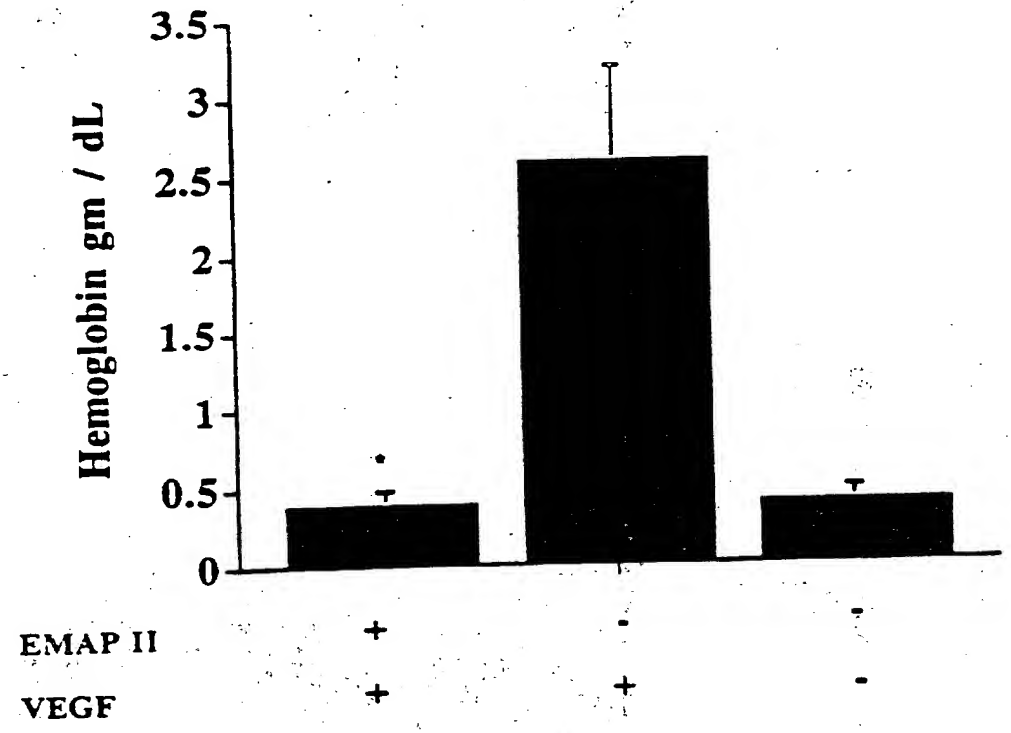


FIG. 11A FIG. 11B

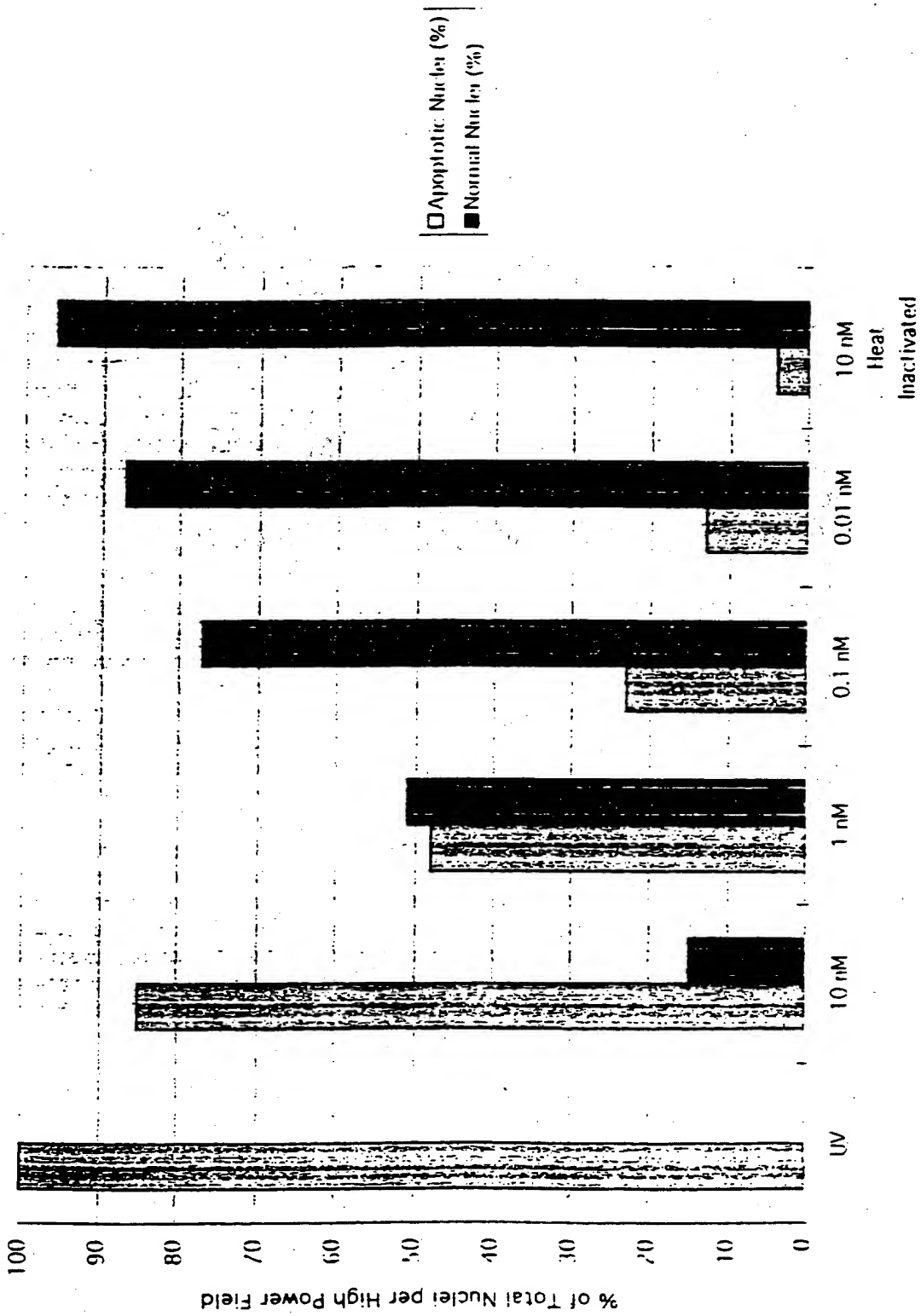
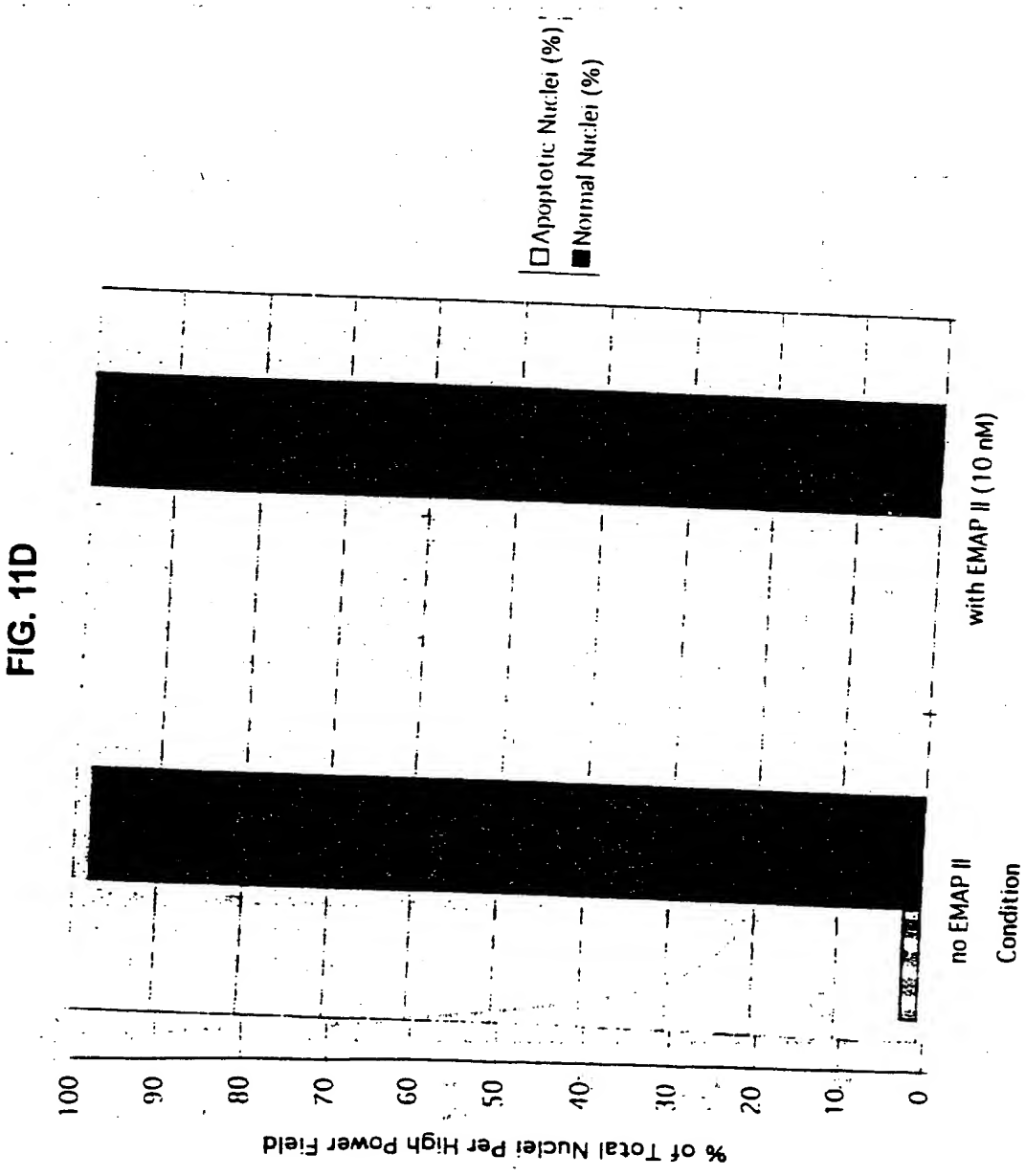

Apoptotic Nuclei (%)
Normal Nuclei (%)
% of Total Nuclei per High Power Field
100
90
80
70
60
50
40
30
20
10
0
UV
10 nM
1 nM
0.1 nM
0.01 nM
10 nM
Heat
Inactivated
EMAP II Concentration

FIG. 11C

FIG. 11D



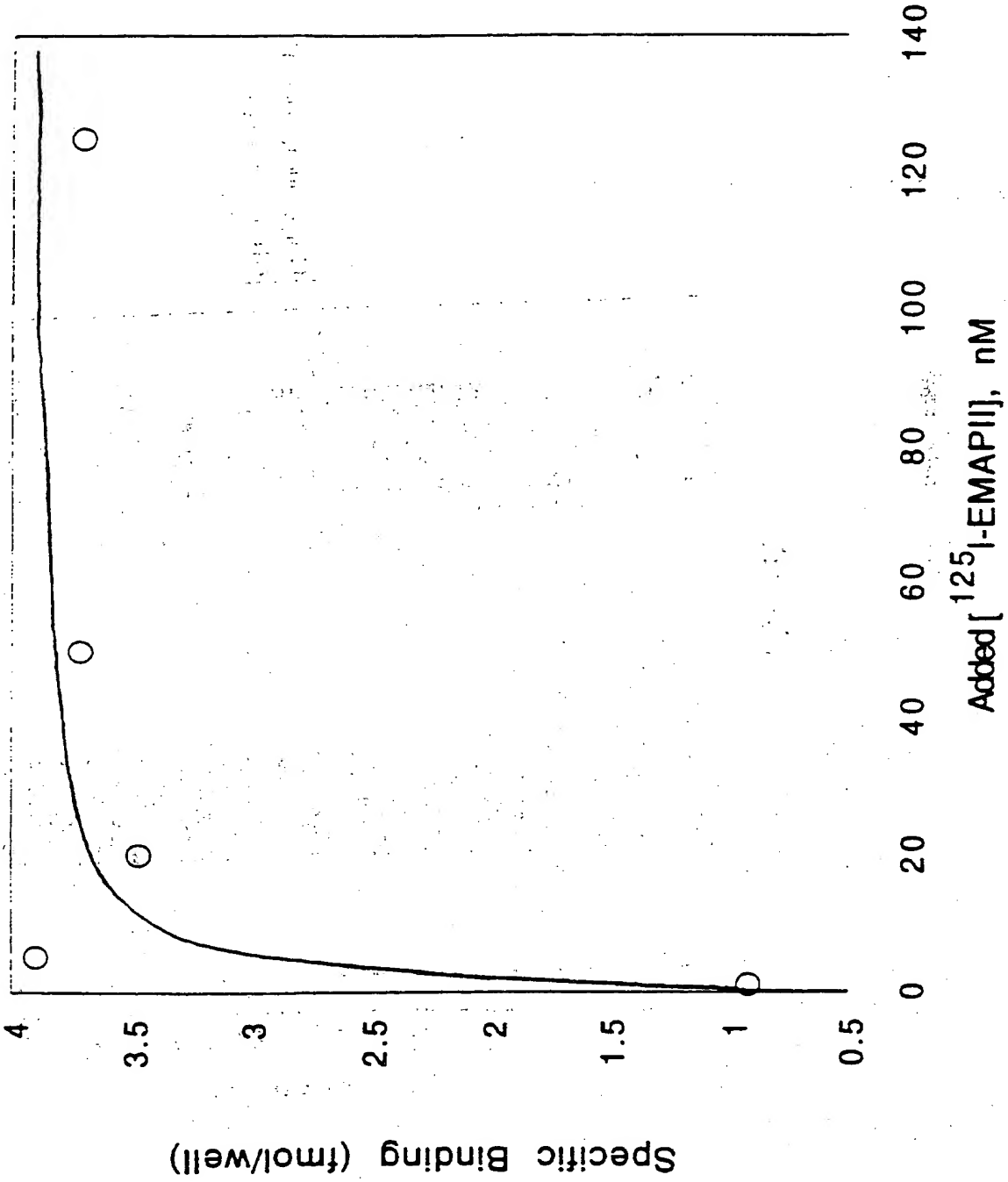


FIG. 11E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/15007

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/17; C12Q 1/02
US CL :435/29; 514/12, 21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/29; 514/2, 12, 21; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG
search terms: emap, endothelial monocyte activating, angiogenesis, neovascularization, retinopathy, endothelial

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KAO et al. Endothelial Monocyte-activating Polypeptide II. The Journal Of Biological Chemistry. 05 October 1992, Volume 267, Number 28, pages 20239-20247.	1-52
X	KAO et al. Characterization of a Novel Tumor-derived Cytokine. The Journal Of Biological Chemistry. 07 October 1994, Volume 269, Number 40, pages 25106-25119, especially page 25108, column 2, last paragraph, page 25109.	23-37, 51, 52
X	US 5,382,514 A (PASSANITI et al.) 17 January 1995, see entire document, especially column 3, lines 50-64, column 11, lines 5-18.	49-52

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search
05 NOVEMBER 1996

Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/15007

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,019,556 A (SHAPIRO et al.) 28 May 1991, see entire document, especially column 8, line 40 through column 10, line 16.	1-12, 21-29, 38, 45-47, 51, 52
X	US 5,198,423 A (TAGUCHI et al.) 30 March 1993, see entire document, especially column 14, lines 30-50.	1-12, 21-29, 38, 45-47, 50-52
X	US 5,202,116 A (BROWN et al.) 13 April 1993, see entire document, especially column 19, lines 20-33.	49
X	US 5,284,827 A (MAIONE et al.) 08 February 1994, see entire document, especially column 4, lines 19-20, column 9, line 50 through column 10, line 37, column 13, line 38 through column 14, line 63.	1-12, 21-29, 38-47, 49-52
X,P	SCHWARZ et al. Endothelial-Monocyte Activating Polypeptide (EMAP) II, a Novel Antiangiogenic Protein, Suppresses Tumor Growth and Induces Apoptosis in Endothelial Cells. American Heart Association Supplement to Circulation. 15 October 1995, Volume 92, Number 8, Supplement, pages I-7 - I-8, abstract no. 0034, see entire abstract.	1-38, 45-52
X	WO 95/09180 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 06 April 1995, see entire document, especially page 6, lines 22-34, page 29, line 4 through page 31, line 9; page 79, lines 10-21, page 113, lines 24-29, claim 68.	1-38, 45-48, 51, 52

PCT

CORRECTED VERSION*

PCT

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International Bureau

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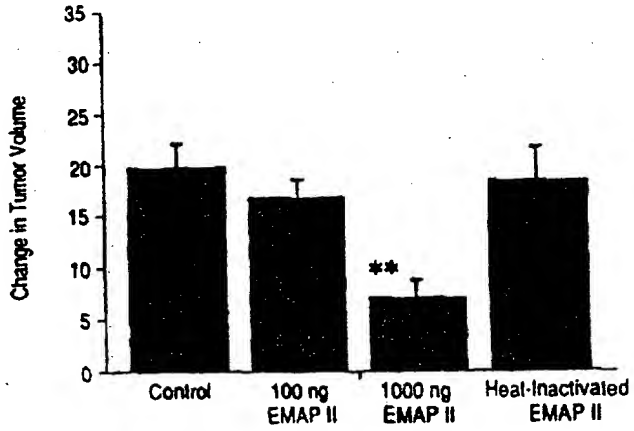
(81) Designated States: AU, CA, JP, MX, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: ANTIANGIOGENIC PROPERTIES OF ENDOTHELIAL-MONOCYTE ACTIVATING POLYPEPTIDE II

(57) Abstract

Tumors are treated by subcutaneously, intraperitoneally or intravenously administering endothelial monocyte activating polypeptide II (EMAP II) or an EMAP II-derived polypeptide. In addition, conditions involving the presence of excess blood vessels, for example retinopathy, are treated with EMAP II or an EMAP II-derived polypeptide.



* (Referred to in PCT Gazette No. 25/1997, Section II)

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ANTIANGIOGENIC PROPERTIES OF ENDOTHELIAL-MONOCYTE ACTIVATING POLYPEPTIDE II

This application claims the benefit of U.S. Provisional No. 60/003,898, filed September 18, 1995, the contents of which are hereby incorporated by reference into the present application.

The invention disclosed herein was made with Government support under PHS Grant Nos. HL42833, HL42507, PERC, from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various references are referred to within parenthesis. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the sequence listing and the claims, or in the body of the text.

Background of the Invention

Recent studies have focussed attention on tumor neovasculature as a critical regulator of the growth of both primary and metastatic neoplastic lesion (Fidler I., 1994; Folkman J., 1989; Folkman J., 1995). Earlier studies emphasized the role of angiogenic factors, such as vascular endothelial growth factor (VEGF) (Plate K., 1992; Berkman, R.; Warren R., 1995; Kim J., 1993), acidic fibroblast growth factor (Maciag T., 1984), basic fibroblast growth factor (bFGF) (Shing Y., 1984), and angiogenin (Fett J., 1985; King T., 1991; Olson K., 1994), in promoting tumor growth and establishment of metastases. For example, in a transgenic murine model, a switch in phenotype from pancreatic adenoma to malignancy was closely tied to expression of angiogenic mediators (Kandel J., 1991), and blocking antibody to VEGF inhibited growth of explanted human tumors in athymic mice (Warren R., 1995; Kim J., 1993). Similar inhibition of experimental tumor growth has also been observed with

antibodies to angiogenin (Olson K., 1994) and bFGF (Hori A., 1991). Alternatively, recent work has delineated endogenous peptides with antiangiogenic activities, including angiostatin (O'Reilly M., 1994), thrombospondin (Dameron K.,1994) and glioma-derived angiogenesis inhibitory factor (Van Meir., 1994). Their presence appears to negatively impact on tumor growth either at the primary tumor site (thrombospondin) or at a site of distant metastases (angiostatin). Formation of the tumor vascular bed, as well as blood vessel formation in other situations, such as ischemia and atherosclerotic plaques (Shweiki, D., 1992; Knighton D., 1983; Kuwabara K., 1995; Sharma H., 1992; Chia M. 1991; Brogi E., 1993), is presumably controlled by the interaction of such positive and negative stimuli on endothelium in diverse vascular beds.

Endothelial-Monocyte Activating Polypeptide II (EMAP II) is a ~20kDa protein isolated from Meth A fibrosarcoma cells (Kao J., 1992; Kao J., 1994), whose tumors exhibit characteristic vascular insufficiency manifested by heterogeneous pattern of thrombohemorrhage and central necrosis (Old L., 1986; Old L., 1961). EMAP II has been described in PCT International Publication No. WO 95/09180, published April 6, 1995, the contents of which are hereby incorporated by reference. These studies show that EMAP II has anti-angiogenic properties and results in suppression of tumor growth, likely due to perivascular apoptotic tissue injury and targeting of EMAP II to proliferating endothelial cells. These results demonstrate that endogenous or exogenously administered EMAP II controls blood vessel formation in a range of pathophysiologically relevant situations.

International Publication No. WO 95/09180 discloses that EMAP II administered in one intratumoral dose followed by one intravenous dose reduces the size of a tumor. WO 95/09180 also discloses that EMAP II has inflammatory activity. On the basis of its inflammatory activity one

would have expected that EMAP II would be toxic and therefore inappropriate for multiple administrations over a long period of time. Surprisingly, it has been found that multiple administrations of EMAP II decrease tumor size even without an intratumoral dose and without observed toxic effect. The ability to administer a therapeutically effective regimen of EMAP II without an intratumoral injection makes it possible to treat tumors whose small size makes it difficult or impossible to administer an intratumoral injection.

Retinal neovascularization is a major cause of blindness in the United States. Pathologic retinal angiogenesis is a common pathway leading to vision loss in disease processes such as retinopathy of prematurity, diabetic retinopathy, sickle cell retinopathy, and age related macular degeneration. Factors associated with retinopathy vascularization include hypoxia (cause of retinopathy of prematurity), diabetes, and known angiogenic factors such as Vascular endothelial growth factor (VEGF). The use of an established model of hypoxic induced retinopathy (Pierce, E. Jan. 1995; Smith, L., Jan. 1994) demonstrates that EMAP II, a protein associated with tumor antiangiogenesis, inhibits the neovascularization associated with retinopathy.

Summary of the Invention

This invention provides a method of treating a tumor in a subject, comprising administering to the subject an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to treat the tumor, wherein the endothelial monocyte activating polypeptide II is administered subcutaneously, intraperitoneally, or intravenously.

This invention provides a method of inhibiting the growth of endothelial cells, comprising contacting the endothelial cells with an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to inhibit growth of the endothelial cells.

Description of the Figures

Figure 1. SDS-PAGE of recombinant EMAP II. E. coli homogenate and pools of fractions containing EMAP II (See Fig. 2, below) were subjected to reduced SDS-PAGE (10-20% Tricine gels; 1-2 μg/lane) and protein visualized by silver staining. Lane 1, E. coli cell homogenate after centrifugation (12,000xg); lane 2, polyethylene imine supernatant; lane 3, Heparin Sepharose pool; lane 4, SP Sepharose pool; lane 5, Phenyltoyopearl pool; and lane 6, EMAP II formulated into PBS.

Figures 2A, 2B and 2C. Chromatographic steps in the purification of recombinant EMAP II. Fig. 2A. Heparin Sepharose. The polyethylene imine supernatant was applied to Heparin Sepharose in Tris buffer, washed and eluted with an ascending salt gradient. Fractions were monitored for absorbance at 280 nm and analyzed on SDS-PAGE and/or immunoblotting to identify the EMAP II pool (designated by the arrow and labeled EMAP II). (Fig. 2B) SP Sepharose. The Heparin Sepharose pool from (Fig. 2A) was concentrated, desalted and applied to SP Sepharose High Performance in MOPS buffer. After washing, EMAP II was eluted by an ascending salt gradient and pooled as above. Phenyl toyopearl. The SP Sepharose pool was adjusted to 2 M $(NH_4)_2SO_4$ and applied to Phenyl toyopearl in phosphate buffer with salt, washed, and EMAP II eluted with a descending salt gradient. The salt gradients are shown as (---), 0-1 M in NaCl (A-B), and (---)0-2 M $(NH_4)_2SO_4$ (C). Absorption at 280nm is shown by the solid line in each figure.

Figures 3A, 3B, 3C , 3D and 3E. Matrigel angiogenesis model: effect of EMAP II on bFGF-induced neovascularization. Mice received subcutaneous Matrigel implants and were sacrificed after 14 days to analyze new vessel formation by histologic examination and hemoglobin assay. Fig. 3A and 3C implant containing bFGF (100 μg/ml)/heparin (40 U/ml) shown at low and high power, respectively; Fig. 3B and 3D, implant containing bFGF/heparin + EMAP II (100 ng/ml) shown at low

and high power, respectively; Fig. 3E, results of hemoglobin (reported as percent of control, Matrigel with vehicle, arbitrarily defined as 100%). Results were evaluated by student t-test and $p<0.001$ comparing hemoglobin levels in the presence of EMAP II with control and bFGF. Magnification in Figures 3A-3C, 10x.

Figures 4A, 4B and 4C. Disappearance of ^{125}I-EMAP II from mouse plasma after IV or IP injection (Fig. 4A), precipitability of the tracer in trichloroacetic acid (Fig. 4B), and tissue accumulation (Fig. 4C). Fig. 4A. Mice received ^{125}I-EMAP II (0.26 μg) by either IV or IP injection and plasma was sampled at the indicated time points. The methods for data fitting and parameters of clearance are described in the text. Fig. 4B. Trichloroacetic acid precipitability of ^{125}I-EMAP II in spleen and B16 tumor harvested 12 hrs after IP injection as above. Tissue was homogenized, weighed, counted, and subjected to precipitation in trichloroacetic acid (20%). The mean ±SD is shown (n=12), and data were analyzed by student t-test; $p<0.001$. Fig. 4C. Deposition ^{125}I-EMAP II in tumor, spleen, brain and liver. Animals received IP ^{125}I-EMAP II, as above at time 0, and then 1 hr prior to harvest ^{51}Cr-labeled microspheres were infused IV. At the 12 hr point, animals were sacrificed, the indicated organs were removed, dried, weighed and counted. The mean ±SE is shown (n=4) and data analyzed by Mann-Whitney showed a $p<0.02$ comparing tissue counts in the tumor to spleen and brain.

Figures 5A, 5B, 5C, 5D, 5E, 5F and 5G. Effect of EMAP II on Lewis Lung Carcinoma (LLC). Mice were injected subcutaneously on day 1 with LLC cells, and then on days 3-15 received every 12 hrs IP either: vehicle alone (control), EMAP II (100 or 1000 ng) or heat-inactivated EMAP II (1000 ng). Fig. 5A. Change in tumor volume in each of the four groups (mean ± SD) is shown (n=40), and data analysis was performed as described in the text ($p<0.034$, Kruskal-Wallis; and $p<0.003$ Mann-Whitney). Figs. 5B-5E. Histology of LLC

tumors harvested from the indicated above groups on day 15: Figure 5B and 5C, vehicle alone, high and low power, respectively; Figure 50C and 5E, EMAP II (100 ng) high and low power, respectively; Figs. 5F-G. DNA fragmentation by in situ nick translation: Fig. 5F, vehicle alone and Fig. 5G, EMAP II (1000 ng).

Figures 6A, 6B, 6C, 6D, 6E and 6F. Effect of EMAP II on cultured endothelial cells (ECs). Figures 6A-6D. Effect on EC monolayer wound repair in vitro. A postconfluent monolayer of ECs was wounded (wound margin at upper right), and then either vehicle (Figures 6A, 6C) or rEMAP II (10 ng/ml; Figures 16B, 16D) was added. After 24 hrs of incubation, cultures were stained with rhodamine phalloidin (Figures 6A-6B) to display the actin-based cytoskeleton or with DAP-1 (Figures 6C-6D) to demonstrate the presence of apoptotic bodies, noted by arrows in Figure 6D. Figures 6E-6F. DNA fragmentation by ELISA of subconfluent ECs in normoxia or hypoxia (pO_2 ≈14 torr), exposed to rEMAP II as indicated. Data shown represent mean and, in each case, S.E. was less than 10%. These experiments were repeated a minimum of three times. Magnification: Figures 6A-6D, 128X.

Figures 7. PCR analysis of EMAP II transcripts in normal murine tissue. RNA was harvested from normal murine tissues as indicated, and processed for PCR as described in the text. The bands corresponding to the amplicons for EMAP II (400 bp) and ß-actin (560 bp) are shown by the arrows. A 100 bp ladder was used as the standard in the far left lane.

Figures 8A, 8B, 8C, 8D and 8E. Lung metastasis model with LLC. Mice received LLC cells subcutaneously and were observed until tumors reached a volume of ≥1.5 cm^3, at which time animals were treated with rEMAP II (1000 ng; IP every 12 hrs; N=8) or vehicle alone (control; N=6) for 72 hrs. Tumors were subsequently resected (there were no local recurrences), and the same treatment regimen was continued for the duration of the study, an additional 15 days. India

ink was instilled intratracheally to enhance visualization of metastases (pale areas) compared with normal tissue (dark areas). Gross appearance of lungs demonstrated many surface macrometastases in controls (Figure 8A) versus their marked suppression in rEMAP II-treated mice (Figure 8B). Histologic examination confirmed this impression (Figure 8C, vehicle-treated, and Figure 8D, EMAP II-treated; arrow in Figure 8D and Figure 8D inset indicate the presence of micrometastasis). In Figure 8E, surface lung metastases/nodules data from all animals was analyzed using Mann-Whitney ($p<0.009$); total surface metastases are shown in the Figure 8E (mean ± S.E.) and surface macrometastases (>2mm; mean ± S.E. for control and EMAP II-treated groups were 80±12.5% and 20±13%, respectively), counted using a calibrated ocular, are shown in Figure 8E (by student t-test $p<0.002$). These experiments were repeated four times. Marker bar, 1 cm (Figures 8A-8B); magnification Figure 8C-8D, 12.8X; and Figure 8D inset, 32X.

Figures 9A, 9B, 9C, 9D, 9E and 9F. Effect of rEMAP II on C6 gliomas implanted intracranially into rats and subcutaneously into mice. Figures 9A-9D. Intracranial C6 gliomas in rats. Figure 9A. C6 glioma cells were implanted stereotactically as described, and rats were maintained for 10 days, at which time they were divided into eight treatment groups as indicated. Tumor volume was evaluated on day 26 (after 16 days of treatment). ** and * indicate $p<0.0001$ and $p<0.005$, respectively, by Kruskal-Wallis. In Fig. 9A, the mean ±SE is shown. Figure 9B-9C. Intracranial tumors, derived from C6 glioma cells, were harvested from animals treated with vehicle (Fig. 9B and 9D; IT/IP) alone or EMAP II (Fig. 9C and 9E; IT/IP). Sections were stained with hematoxylin and eosin (9B, 9C)) or subjected to the TUNEL procedure (9D, 9E). Figure 9F. Subcutaneous C6 gliomas in nude mice. Tumor cells were implanted, animals were maintained for 3 days, and treatment with EMAP II was initiated for the next 24 days as described. At the end of the experiment, tumor volume was measured and data shown

represent the mean ± SE. The intracranial tumor experiments were repeated three times and the subcutaneous tumor studies were repeated twice.

Figures 10A, 10B, 10C, 10D and 10E. Effect of rEMAP II on vascular ingrowth into Matrigel implants impregnated with VEGF. Matrigel mixtures containing VEGF (100 ng/ml) were administered subcutaneously and, simultaneously, animals (N = 10 per group) received rEMAP II (1 μg; IP every 12 hrs) in vehicle or vehicle alone for the next 14 days. Implants were evaluated by hematoxylin and eosin staining (Figures 10A & 10C, treated with rEMAP II; Figures 10B & 10D, treated with vehicle) and quantitation of hemoglobin content (Figures 10E; mean ± SE; * indicates $P<0.01$). The experiments were repeated three times.

Figures 11A, 11B, 11C, 11D and 11E. Interaction of rEMAP II with cultured endothelial and C6 glioma cells. Figure 11A-11B. Human umbilical vein endothelial cells or C6 glioma cells in Medium 199 containing fetal calf serum (10%) were exposed to rEMAP II (10nM;A) or medium alone (B) for 24 hrs at 37°C, samples were harvested and subjected to TUNEL analysis as described. Figure 11C. Quantitation of apoptotic endothelial nuclei as a ratio of labelled nuclei/cells counted in each of ten high power fields in the presence of the indicated concentration of rEMAPII. * denotes P<x. 11D. Quantitation of labelled nuclei as in (C) when C6 glimoa cells in Dulbecco's MEM were incubated with the indicated concentration of rEMAPII or medium alone. Figure 11E. Radioligand binding study with ^{125}I-rEMAP II. Human umbilical vein endothelial cell monolayers or C6 glioma cells were incubated with the indicated concentrations of ^{125}I-rEMAP II alone or in the presence of an 100-fold excess of unlabelled rEMAP II. Specific binding is plotted and the curve indicates the best-fit using nonlinear least squares analysis. Parameters of binding on endothelial cells were: Kd= 1.9 nM and B=compared with C6 glioma cells. Similar radioligand

binding experiments on C6 glioma cells showed no specific binding.

Detailed Description

This invention provides a method of treating a tumor in a subject, comprising administering to the subject an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to treat the tumor, wherein the endothelial monocyte activating polypeptide II is administered subcutaneously, intraperitoneally, or intravenously. Many types of tumors can be treated according to this method. In a preferred embodiment the tumor is a carcinoma.

The term "EMAP II" refers to Endothelial Monocyte Activating Polypeptide II. The term "rEMAP II" refers to recombinant Endothelial Monocyte Activating Polypeptide II. EMAP II may also include variants of naturally occurring EMAP II. Such variants can differ from naturally occurring EMAP II in amino acid sequence or in ways that do not involve sequence, or both. Variants in amino acid sequence are produced when one or more amino acids in naturally occurring EMAP II is substituted with a different natural amino acid, an amino acid derivative or non-native amino acid. Particularly preferred variants include naturally occurring EMAP II, or biologically active fragments of naturally occurring EMAP II, whose sequences differ from the wild type sequence by one or more conservative amino acid substitutions, which typically have minimal influence on the secondary structure and hydrophobic nature of the protein or peptide. Variants may also have sequences which differ by one or more non-conservative amino acid substitutions, deletions or insertions which do not abolish the EMAP II biological activity. Conservative substitutions (substituents) typically include the substitution of one amino acid for another with similar characteristics such as substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. The non-polar (hydrophobic)

amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

Other conservative substitutions can be taken from Table 1, and yet others are described by Dayhoff in the Atlas of Protein Sequence and Structure (1988).

Table 1: Conservative Amino Acid Replacements

For Amino Acid	Code	Replace with any of
Alanine	A	D-Ala, Gly, beta-ALa, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, Beta-Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Met, D-Met

Lysine	K	D-Lys,Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val, Norleu
Phenylalanine	F	D-Phe,Tyr, D-Thr,L-Dopa,His,D-His, Trp, D-Trp, Trans 3,4 or 5-phenylproline, cis 3,4 or 5 phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4-carboxylic acid, D- or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O) D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr,Phe, D-Phe, L-Dopa, His,D-His
Valine	V	D-Val, Leu,D-Leu,Ile,D-Ile, Met, D-Met

Other variants within the invention are those with modifications which increase peptide stability. Such variants may contain, for example, one or more non-peptide bonds (which replace the peptide bonds) in the peptide sequence. Also included are: variants that include residues other than naturally occurring L-amino acids, such as D-amino acids or non-naturally occurring or synthetic amino acids such as beta or gamma amino acids and cyclic variants. Incorporation of D- instead of L-amino acids into the polypeptide may increase its resistance to proteases. See, e.g., U.S. Patent 5,219,990.

The peptides of this invention may also be modified by various changes such as insertions, deletions and

substitutions, either conservative or nonconservative where such changes might provide for certain advantages in their use.

In other embodiments, variants with amino acid substitutions which are less conservative may also result in desired derivatives, e.g., by causing changes in charge, conformation and other biological properties. Such substitutions would include for example, substitution of hydrophilic residue for a hydrophobic residue, substitution of a cysteine or proline for another residue, substitution of a residue having a small side chain for a residue having a bulky side chain or substitution of a residue having a net positive charge for a residue having a net negative charge. When the result of a given substitution cannot be predicted with certainty, the derivatives may be readily assayed according to the methods disclosed herein to determine the presence or absence of the desired characteristics.

Variants within the scope of the invention include proteins and peptides with amino acid sequences having at least eighty percent homology with EMAP II. More preferably the sequence homology is at least ninety percent, or at least ninety-five percent.

Just as it is possible to replace substituents of the scaffold, it is also possible to substitute functional groups which decorate the scaffold with groups characterized by similar features. These substitutions will initially be conservative, i.e., the replacement group will have approximately the same size, shape, hydrophobicity and charge as the original group. Non-sequence modifications may include, for example, in vivo or in vitro chemical derivatization of portions of naturally occurring EMAP II, as well as changes in acetylation, methylation, phosphorylation, carboxylation or glycosylation.

In a further embodiment the protein is modified by chemical

modifications in which activity is preserved. For example, the proteins may be amidated, sulfated, singly or multiply halogenated, alkylated, carboxylated, or phosphorylated. The protein may also be singly or multiply acylated, such as with an acetyl group, with a farnesyl moiety, or with a fatty acid, which may be saturated, monounsaturated or polyunsaturated. The fatty acid may also be singly or multiply fluorinated. The invention also includes methionine analogs of the protein, for example the methionine sulfone and methionine sulfoxide analogs. The invention also includes salts of the proteins, such as ammonium salts, including alkyl or aryl ammonium salts, sulfate, hydrogen sulfate, phosphate, hydrogen phosphate, dihydrogen phosphate, thiosulfate, carbonate, bicarbonate, benzoate, sulfonate, thiosulfonate, mesylate, ethyl sulfonate and benzensulfonate salts.

Variants of EMAP II may also include peptidomimetics of EMAP II. Such compounds are well known to those of skill in the art and are produced through the substitution of certain R groups or amino acids in the protein with non-physiological, non-natural replacements. Such substitutions may increase the stability of such compound beyond that of the naturally occurring compound.

In an embodiment of this invention the subject is a mammal. Examples of suitable mammalian subjects include, but are not limited to, murine animals such as mice and rats, hamsters, rabbits, goats, pigs, sheep, cats, dogs, cows, monkeys and humans. In a specific embodiment the agent is administered intraperitoneally.

By means of well-known techniques such as titration and by taking into account the observed pharmacokinetic characteristics of the agent in the individual subject, one of skill in the art can determine an appropriate dosing regimen. See, for example, Benet, et al., "Clinical Pharmacokinetics" in ch. 1 (pp. 20-32) of Goodman and

Gilman's The Pharmacological Basis of Therapeutics, 8th edition, A.G. Gilman, et al. eds. (Pergamon, New York 1990). In an embodiment of this invention the agent is administered in at least twenty doses. In a specific embodiment the agent is administered in about twenty-four doses. In an embodiment the agent is administered over a period of at least ten days. In a specific embodiment, the agent is administered over a period of about twelve days. In an embodiment of this invention, the frequency of administration is at least about one dose every twelve hours. In an embodiment the effective amount is from about 2.4 micrograms to about 24 micrograms. In an embodiment the effective amount is from about 100 nanograms to 24 micrograms per dose. In a more specific embodiment the effective amount is from about 100 nanograms to about 1000 nanograms per dose.

In an embodiment of the method described herein, the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDASRLDLRIG (C/R)IVTAKKHPDADSLYVEEVDVGEAAPRTVVSGLVNHVPLEQMQNRMVVLLCNLK PAKMRGVLSQAMVMCASSPEKVEILAPPNGSVPGDRITFDAFPGEPDKELNPKKKIWE QIQPDLHTNAECVATYKGAPFEVKGKGVCRAQTMANSGIK (SEQ I.D. No. ___), wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues. In a preferred embodiment the homology is at least about ninety-five percent.

In another embodiment of the method described herein the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDVSRLDLRIG (C/R)IITARKHPDADSLYVEEVDVGEIAPRTVVSGLVNHVPLEQMQNRMVILLCNLK PAKMRGVLSQAMVMCASSPEKIEILAPPNGSVPGDRITFDAFPGEPDKELNPKKKIWE QIQPDLHTNDECVATYKGVPFEVKGKGVCRAQTMSNSGIK (SEQ I.D. No. ___), wherein the sequence is truncated by from zero to about

three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues. In a preferred embodiment the homology is at least about ninety-five percent.

In a preferred embodiment of the method described herein the agent is endothelial monocyte activating polypeptide II. In a more specific embodiment, the EMAP II is murine EMAP II or human EMAP II. In an embodiment the endothelial monocyte activating polypeptide II is recombinant endothelial monocyte activating polypeptide II.

An advantage of the above-described method compared to treatment protocols involving intratumor injection is that tumors that are too small for intratumoral injection can be treated before they grow to a larger size. Accordingly, in an embodiment of this invention the tumor is too small for intratumoral injection. For example, in a specific embodiment, the diameter of the tumor is less than or equal to about two millimeters.

This invention provides a method of inhibiting the growth of endothelial cells, comprising contacting the endothelial cells with an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to inhibit growth of the endothelial cells. In an embodiment, the endothelial cells are aortic endothelial cells, for example bovine aortic endothelial cells.

This invention provides a method of inhibiting the formation of blood vessels in a subject, comprising administering to the subject an effective amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, thereby inhibiting the formation of blood vessels in the subject.

In an embodiment of this invention the subject is a mammal. Examples of suitable mammalian subjects include, but are not limited to, murine animals such as mice and rats, hamsters, rabbits, goats, pigs, sheep, cats, dogs, cows, monkeys and humans.

The agent may be administered according to techniques well known to those of skill in the art, including but not limited to subcutaneously, intravascularly, intraperitoneally, topically, or intramuscularly.

In an embodiment the effective amount is from about 10 nanograms to about 24 micrograms. In a specific embodiment the effective amount is from about 100 nanograms to about 1 microgram.

In an embodiment of the method described herein, the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDASRLDLRIG (C/R)IVTAKKHPDADSLYVEEVDVGEAAPRTVVSGLVNHVPLEQMQNRMVVLLCNLK PAKMRGVLSQAMVMCASSPEKVEILAPPNGSVPGDRITFDAFPGEPDKELNPKKKIWE QIQPDLHTNAECVATYKGAPFEVKGKGVCRAQTMANSGIK (SEQ I.D. No. ___), wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues. In a preferred embodiment the homology is at least about ninety-five percent.

In another embodiment of the method described herein the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDVSRLDLRIG (C/R)IITARKHPDADSLYVEEVDVGEIAPRTVVSGLVNHVPLEQMQNRMVILLCNLK PAKMRGVLSQAMVMCASSPEKIEILAPPNGSVPGDRITFDAFPGEPDKELNPKKKIWE QIQPDLHTNDECVATYKGVPFEVKGKGVCRAQTMSNSGIK (SEQ I.D. No. ___), wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one

hundred thirty-six carboxy-terminal residues. In a preferred embodiment the homology is at least about ninety-five percent.

In a preferred embodiment of the method described herein the agent is endothelial monocyte activating polypeptide II. In a more specific embodiment, the EMAP II is murine EMAP II or human EMAP II. In an embodiment the endothelial monocyte activating polypeptide II is recombinant endothelial monocyte activating polypeptide II.

This invention provides a method of treating a condition involving the presence of excess blood vessels in a subject, comprising administering to the subject an effective amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, thereby treating the condition involving the presence of excess blood vessels.

In an embodiment, the condition involves the presence of excess blood vessels in the eye. One such condition is retinopathy. In specific embodiments of the method the retinopathy is diabetic retinopathy, sickle cell retinopathy, retinopathy of prematurity, or age related macular degeneration.

The present invention provides for a method of treating a tumor in a subject, comprising administering to the subject an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to treat the tumor, wherein the endothelial monocyte activating polypeptide II is administered subcutaneously or intraperitoneally; and intravenously, intracranially, or intramorally. The tumor may be a glioblastoma. The agent may be administered intratumorally by positive pressure microinfusion.

The present invention further provides for a method for evaluating the ability of an agent to inhibit growth of endothelial cells, which includes: (a) contacting the endothelial cells with an amount of the agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide; (b) determining the growth of the endothelial cells, and (c) comparing the amount of growth of the endothelial cells determined in step (b) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit growth of endothelial cells.

The present invention provides for a method for evaluating the ability of an agent to inhibit the formation of blood vessels in a cellular environment, which comprises: (a) contacting the cellular environment with an amount of the agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide; (b) determining whether or not blood vessels form in the cellular environment, and (c) comparing the amount of growth of blood vessels determined in step (b) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit formation of blood vessels is the cellular environment.

As used herein, a cellular environment includes but is not limited to a cell culture system, cells in vivo, cells in vitro, an organ culture, an animal model system. A cellular environment may include a cells growing in a subject, a tumor cell culture system, an endothelial cell culture system, an embryonic cell culture system, an angiogenic cell culture system. A cellular environment may be either in vitro or in vivo. A cellular environment may include a hybridoma cell culture system.

The present invention provides for a pharmaceutical composition which comprises an agent capable of inhibiting

blood vessel formation and a pharmaceutically acceptable carrier. The carrier may include but is not limited to a diluent, an aerosol, a topical carrier, an aquous solution, a nonaqueous solution or a solid carrier.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

ANTI-TUMOR TREATMENT

Example 1: Endothelial-Monocyte Activating Polypeptide II, A Novel Antiangiogenic Protein, Suppresses Tumor Growth and Induces Apoptosis of Growing Endothelial Cells.

Materials and Methods

Cell culture. Bovine aortic endothelial cells (ECs) were isolated from calf aortae, grown in culture and characterized, based on the presence of von Willebrand factor and thrombomodulin, as described previously (Nawroth P., 1988). Bovine vascular smooth muscle cells were prepared by additional scraping of the aortae following removal of the endothelium, and were characterized based on the presence of smooth muscle cell actin (Gown A., 1985). Lewis Lung carcinoma cells (LLC), obtained from American Type Culture Collection (ATCC), NIH 3T3 cells (ATCC), and B16 (F10) cells were all maintained in high glucose defined minimal essential medium (DMEM; Gibco) containing fetal bovine serum. Meth A tumor cells were provided by Dr. Lloyd Old (Center for Cancer Research, NY), and grown as described (Old L., 1987; Old L., 1961).

Preparation of recombinant murine EMAP II and EMAP II ELISA. Recombinant EMAP II was prepared from E. coli (host HMS174[DE3]) transformed with a plasmid containing the coding sequence for mature EMAP II, as described previously (Kao J., 1994). A procedure was developed for purification of recombinant EMAP II on a preparative scale. Frozen (-80°C) E. coli cell paste was mixed 1:10 (w/v) with Tris-HCl (20mM; pH 7.4) containing octyl-ß-glucoside (0.1%) and an homogeneous suspension formed by agitation with a TURRAX® for 20 min (speed 60) at 4°C. The suspension was then disrupted by three passes through a TURRAX® Microfluidizer (Mode 110F) at 4°C. Polyethylene imine at pH 7 was then added to the homogenate to a concentration of 0.25%. The homogenate was left for 30 min on ice to precipitate cell debris and DNA. Solids were removed from the homogenate by centrifugation (5000xg; 30 min), the polyethylene imine

supernatant was retained and filtered (0.2 μm), and applied (3 mls sample ml of gel) to Heparin sepharose CL-4B (Pharmacia, Piscataway NJ; 120 ml bed volume) equilibrated in Tris-HCl (20 mM; pH 7.4) containing octyl-ß-glucoside (0.1%). After washing the column with the same buffer (20 ml/min), a linear ascending NaCl gradient (0 to 0.5M) in the wash buffer was applied. Fractions were pooled on the basis of purity by silver stained SDS-PAGE and by immunoblotting with antibodies prepared to the N-terminus of mature EMAP II (Kao J., 1992). EMAP II biological activity was measured based on induction of tissue factor in cultural endothelial cell, as described (Kao J., 1992).

The Heparin Sepharose pool was concentrated using an Amicon Stirred Cell (Amicon) with an Amicon YM10 Diaflo Ultrafilter to less than 100 ml. The retentate was desalted into 3-(Morpholino)-propane-sulfonic acid (MOPS; 25 mM; pH 6.9) on a Sephadex G25 (Medium Grade, Pharmacia) size exclusion column (480 ml bed volume). The pool was then applied to an SP Sepharose High Performance (Pharmacia) cation exchange column (55ml bed volume) run at a flow rate of 10 ml/min. After washing the column with MOPS buffer, EMAP II-containing fractions were eluted by application of a 0 to 0.5 M ascending linear salt gradient in MOPS. The pool was identified, assayed for total protein an biological activity as above.

EMAP II-containing fractions from SP Sepharose chromatography were adjusted to 2 M in $(NH_4)_2SO_4$ with solid $(NH_4)_2SO_4$ and applied to a Phenyl Toyopearl 650 M (Tosohaas) column (90 ml bed volume) equilibrated in sodium phosphate (20 mM; pH 7) containing 1 M $(NH_4)_2SO_4$. After washing with the above buffer, a descending gradient of salt (2 to 0M) in sodium phosphate (20 mM) was applied. EMAP II-containing fractions were pool and characterized as above.

EMAP II from in the Phenyl Toyopearl column eluate was concentrated to 3-5 mg/ml, and formulated into phosphate-

buffered saline (PBS; pH 7.4) by buffer exchange on a Sephadex G25 column (as above). Lipopolysaccharide (LPS) was removed using filtration through a Posidyne filter (Pall Corp.), and LPS levels were estimated using the Endospecy chromogenic assay (limit of detection <10pg/ml). Purified EMAP II, as well as EMAP II in fractions obtained during the purification procedure was subjected to N-terminal sequence analysis, mass spectrometry and SDS-PAGE. Samples were mixed with 1:0.5 (v:v) of Tricine SDS sample buffer with the addition of 1/10 volume of 1 M dithiothreitol, boiled for 5 min, and applied (1 μg/lane) to 10-20% Tricine gels (Novex) and electrophoresed in SDS Tricine running buffer at constant voltage (100 V) for about 2 hrs at room temperature. Protein was visualized by silver staining, and molecular weight markers (Novex) were run simultaneously. Immunoblotting was performed following SDS-PAGE by transferring protein to nitrocellulose in Tris-HCl (12 mM), glycine (96 mM; final pH 8.3) containing methanol (20%) using the Novex Western Transfer Apparatus at constant voltage (30 V) for 2-4 hr (4°C). Prestained, low molecular weight markers (Bio-Rad) were used to follow the transfer. Immunoreactive protein was visualized using rabbit anti-mature EMAP II N-terminal peptide IgG (0.1 μg/ml) followed by the Amplified Alkaline Phosphatase Goat Anti-Rabbit Immuno-Blot Assay Kit (Bio-Rad).

Antibody to EMAP II was prepared by standard methods (30), and was found to be monospecfic based on immunoblotting of plasma and cell extracts. This antibody was used to develop an ELISA to detect EMAP II antigen; cells or tissues were homogenized in the presence of protese inhibitors (phenylmethylsulfonyl fluoride, 1 mM; trasylol, 0.1%), centrifuged to remove debris, and the supernatant was diluted in carbonate/bicarbonate buffer (pH 9.6) and incubated in Maxisorb microtiter plates (Nunc) overnight at 4°C. Wells were washed with phosphate-buffered saline, excess sites were blocked with bovine serum albumin (1%) in phosphate-buffered saline for 30 min at room temperature,

and then incubated for 1 hr at 37°C with monospecific polyconal rabbit immune IgG against EMAP II dissolved in phosphate-buffered saline containing bovine serum albumin (1%). Primary antibody was revealed with peroxidase conjugated secondary antibody and o-phenylenediamine dihydrchloride as the chromogenic substrate. Concentrations of EMAP II were determined by comparison with a standard curve made with known amounts of recombinant murine EMAP II.

Matrigel model. Matrigel (Kleinman H., 1986; Passaniti A., 1992) (Collaborative Research) containing either EMAP II (100 ng/ml), bFGF (100 ng/ml) (Collaborative Research) and heparin (40 U/ml; Sigma), EMAP II (100 ng) + bFGF/heparin, vehicle alone (1% BSA), or heat-inactivated EMAP II (alone or with bFGF/heparin) were mixed at 4°C. Matrigel mixtures were injected subcutaneously into C57BL6/J mice (0.25 ml/site) at two sites per animal. The angiogenic response was analyzed at 7 and 14 days post-inoculation by routine histology and hemoglobin assay (Sigma).

Murine clearance studies. Clearance of EMAP II in mice was assessed using ^{125}I-labelled EMAP II. EMAP II was radioiodinated by the Bolton and Hunter method (3.2 mol of ester/mol of protein) (Bolton A., 1973), and the tracer was 99% precipitable in trichloroacetic acid (20%), migrated as a single band with Mr ≈20 kDa on SDS-PAGE, and had a specific radioactivity of ≈8000 cpm/ng. Balb/c mice received ^{125}I-EMAP II (0.26 μg) either intravenously (IV) via the tail vein or intraperitoneally (IP). Plasma samples were taken at the indicated times, and animals were sacrificed at 24 hours. Organs were then dried, weighed and radioactivity assessed. In addition, C57BL6/J mice bearing 14 day old subcutaneous B16 tumors received ^{125}I-EMAP II (0.26 μg/animal; IP), and 1 hour before sacrifice ^{51}Cr-labelled microspheres (10 μ) in normal saline were infused (the latter to monitor residual blood in the tissue). These studies were performed to define ^{125}I-EMAP II plasma clearance, volume of distribution, and accumulation in tumor

tissue. In each case, tissue associated radioactivity was determined on weighed samples either after drying (for total radioactivity), or following homogenization of tissue and trichloroacetic acid precipitation (20%). ^{125}I-EMAP II in the tissue was corrected for residual blood based on the presence of ^{51}Cr-labelled microsperes. Plasma ^{125}I-EMAP II concentration data were fit to a two-compartment open model using nonlinear regression by extended leat squares analysis (Siphar, SIMED, Creteil, France). In order to assess the "goodness of fit," residual analysis (an examination of the standard deviation) was performed. In addition to the Likelihood test, Akaike, Leonard and Schwarz criteria were tested to select the most appropriate model (Yamoaka K., 1978). $t_{1/2}\alpha, t_{1/2}\beta, t_{1/2}r$ denote half-lives for distribution, elimination and resorption half-lives, respectively.

Tumor models. LLC and B16 (F10) cells were rinsed with Hanks buffered saline solution, trypsinized, counted, resuspended in phosphate-buffered saline, and injected subcutaneously into backs of C57BL6/J mice (2×10^6 cells/animal). On the third day following administration of tumor cells, animals underwent IP injection of EMAP II every 12 hrs for 12 days of either vehicle alone (serum albumin, 1%), vehicle + EMAP II (at 100 or 1000 ng), or vehicle + heat-inactivated EMAP II (1000 ng). Tumor growth was assessed with calipers every third day (from days 3-15), and tumor volume was calculated according to the formula for a spehircal segment (35), $V = \pi h (h^2+3a^2)/6$, where h= height of the segment, a= (length+width)/2, and V= volume. Tumor volume data was analyzed using the Kruskal-Wallis one way ANOVA and a Mann-Whitney mean rank test. Animals were sacrificed and tumors analyzed histologically at day 15.

Histologic analysis was performed on formalin fixed, paraffin embedded tissue, using hematoxylin and eosin staining. DNA nick translation was used in tumor tissue (LLC an Meth A) to evaluate apoptosis. Paraffin embedded tumor slices were deparaffinized and digoxigenin-11-UTP was

used to label fragmented DNA according to the Genius 1 kit (Amersham, location). In brief, tissue was treated with proteinase K (1 μg/ml), and incubated with digoxigenin-11-UTP, klenow, and DNTP's overnight. Nitroblue tetrazolium and alkaline phosphatase were used to reveal the digoxigenin labelled DNA fragments. In addition, sequential sections of Meth A tumors underwent analysis for DNA fragmentation and EMAP II. Apoptosis was analyzed in cultured cells exposed to EMAP II (10 and 100 ng). Cultures were exposed to hypoxia (pO_2 ≈14 torr) using a specially constructed controlled environment chamber, as described previously (Ogawa S., 1990). Cells were incubated with EMAP II, as indicated, and were then fixed in paraformaldahyde (2.5%), rinsed with phosphate-buffered saline, incubated with 6-diamidino-2-phenylindoledilactate (DAP-1; final concentration, 1 ng/ml) and mounted with glycerol (10%). DNA laddering for apoptosis in cultured cells exposed to EMAP II was performed as described (Gorczyca W., 1993). Briefly, after 12 hours of exposure to EMAP II, cultures were treated with lysis buffer (Tris-borate buffer, 45 mM; EDTA, 1mM; pH 8.0; NP-40, 0.25%), digested with proteinase K (1 mg/ml) and RNAase A (0.1 mg/ml), and then DNA was purified by phenol-chloroform extraction. DNA (10 μg/lane) was subjected to agarose gel (1.8%) electrophoresis at 40 volts using an 100 bp ladder as standard (Boehringer Mannheim). Gels were stained with ethidium bromide.

Results

Preparative scale purification of recombinant EMAP II.

Previous studies have employed FPLC Mono Q followed by preparative SDS-PAGE to prepare homogenous EMAP II (Kao J., 1992; Kao J., 1994), resulting in microgram quantities of purified EMAP II. In order to obtain the larger amounts of polypeptide necessary for a range of studies to characterize properties of EMAP II, using in vitro and in vivo systems, a larger scale approach was employed. E. coli paste, from bacteria transformed with the a plasmid expressing mature EMAP II, was disrupted using a microfluidizer, the latter

method found to be 100% effective based on light microscopy. Addition of polyethylene imine to a final concentration of 0.25% removed many of the contaminating polypeptide bands (Fig. 1, compare lanes 1 and 2) from the homogenate. Application of the polyethylene imine supernatant (800 mg) to Heparin Sepharose followed by elution with an ascending salt gradient yielded a pool of protein (150 mg) significantly enriched in EMAP II (Fig. 2A) and containing only minor contaminants by SDS-PAGE (Fig. 1, lane 3). The majority of contaminating protein eluted in the flow through of the column or at the beginning of the salt gradient (Fig. 2A). Protein yields of over 90% were obtained from the concentration and buffer exchange of the Heparin Sepharose pool.

SP Sepharose High Performance chromatography of the EMAP II-rich pool from Heparin Sepharose (130 mg) further removed contaminating proteins (Fig. 2B), shown to be principally the high molecular weight contaminants, as judged by SDS-PAGE (Fig. 1, lane 4). The latter more slowly migrating polypeptide bands were subjected to N-terminal sequence analysis and shown to be of bacterial origin and unrelated to EMAP II or the transformation procedure. EMAP II-containing fraction eluted at approximately 0.15 M sodium chloride in the gradient, and protein yields of over 90% were obtained for this step. Final purification of the SP Sepharose pool (130 mg) was achieved by Phenyl Toyopearl Chromatography (Fig. 2C) which removed any residual contaminating non-EMAP II bands (Fig. 1, lane 5). EMAP II-containing fractions eluted at approximately 1 M $(NH_4)_2SO_4$ and yields of nearly 100% were obtained.

Protein yields of over 98% were obtained by the concentration and formulation of EMAP II into phosphate-buffered saline. Posidyne filtration caused no loss of protein and reduced endoxoin levels to <10 pg/3-5 mg purified EMAP II protein. The final formulated pool was seen as an apparently diffuse band at 21 kDa by gel

electrophoresis (Fig. 1, lane 6). The faint band at Mr ≈40 kDa was probably due to aggregation of EMAP II, as indicated by the characterization of the purified material below. Mass spectrometry gave measured mass of 18,006 which is close to the expected mass of 17,970. N-terminal sequence analysis showed a single sequence with an 100% match between purified murine EMAP II and the published sequence (Kao J., 1992; Kao J., 1994). The purified material was also recognized by anti-mature EMAP II amino terminal peptide IgG by immunoblotting, and in the endothelial cell tissue factor induction assay gave activities of 0.3-0.4 units/ng of protein. The latter is what has been observed with nonrecombinant EMAP II prepared from meth A-induced murine fibrosarcomas (Kao J., 1992) or recombinant EMAP II prepared by a non-preparative scale method (Kao J., 1994). The faint band at Mr ≈40kDa observed on SDS-PAGE of purified (r)EMAP II preparations (Fig. 1, lane 6), was thus most likely to represent aggregates (identical N-terminal sequence and immunoreactive with anti-mature EMAP II-N-terminal peptide IgG (Kao J., 1992) on immunoblotting. Heat-treated EMAP II was boiled for 15 min, and had no activity with respect to previously described effects of EMAP II on ECs or mononuclear phagocytes (Kao J., 1992; Kao J. 1994).

Effect of EMAP II on bFGF-induced angiogenesis. To evaluate the ability of EMAP II to regulate blood vessel formation in response to known growth factor, bFGF and herapin were mixed with a gel of basement membrane proteins produced by Engelbreth-Holm-Swarm tumor cells (Matrigel) to serve as a model angiogenic stimulus (Kleinman H., 1986; Passaniti A., 1992). Subcutaneous Matrigel implants in C57BL6/J mice were evaluated 14 days after inoculation for vessel formation, cellular infiltration and hemoglobin content. Histologic analysis of the gel showed formation of vessel and white cell infiltration to be most pronounced in implants from animals treated with bFGF and heparin (Fig. 3A), which closely parallelled the appearance of implants from animals whose gel content either bFGF/herapin + vehicle (albumin) or

bFGF/herapin + heat-treated EMAP II (Fig. 3B). This induction of blood vessel formation is similar to that reported previously with bFGF in this model (Passaniti A., 1992). In contrast, implants from animals treated with bFGF/herapin + EMAP II displayed marked reduction in vessel ingrowth (Fig. 3C); little-to-no vessel formation and only a minimal cellular infiltrate was observed (n=36). Consistent with these histologic results, there was a 76% reduction in hemoglobin content in implants containing EMAP II, compared to vehicle alone (defined as 100%), heat-inactivate EMAP II (89%) or bFGF/herapin (146%) (Fig. 3D).

Plasma clearance and tissue deposition of infused EMAP II.

In order to perform in vivo studies with EMAP II, its plasma clearance and tissue deposition was evaluated (Fig. 4A). Clearance studies were performed using ^{125}I-EMAP II administered either IV or IP. The fall in plasma concentration of) I-EMAP II after IV injection fit best to a bi-exponential function; the distribution and elimination half-lives were 0.47±0.17 and 103±5 min, respectively. Following IP injection, ^{125}I-EMAP II was detected in plasma after 1 min, and the maximum concentration was reached by 35±10min. The resorption phase of EMAP II handling in vivo was best described as a first-order process. The elimination phase following IP administration fit to a monoexponential decline, and the resorption and elimination half-lives were 50.1±0.10 and 102±6 min, respectively.

Animals bearing B16 tumors were analyzed for tumor-associated radioactivity after receiving an IP injection of ^{125}I-EMAP II; at 1, 6, and 12 hours radioactivity accumulated in the tumor (56±20 cpm/mg tumor tissue; 63±8.9% precipitable in 20% trichloroacetic acid) was 2.5-fold greater than in the spleen (22.7±10 cpm/mg; 39.7±16.7% precipitable in 20 trichloroacetic acid) (Fig. 4B-C; the figure shows data from the 12 hour time point that is consistent with that observed at earlier times). Other normal tissues, such as liver and brain, also showed a lower

amount of radioactivity compared with that present in the tumor (Fig. 4C).

Effect of EMAP II on growth of primary tumors. Mice implanted subcutaneously with LLC cells developed tumors which showed a marked reduction in size when animals received EMAP II, versus controls with vehicle alone or vehicle + heat-treated EMAP II (Fig. 5A). These differences were statistically significant using either the Kruskal-Wallis one way ANOVA analysis ($p<0.034$) or by Mann-Whitney analysis ($p<0.003$) (Fig. 5A). Histologic study of LLC tumors allowed to grow for 15 days and injected every 12 hrs with vehicle (albumin 1%) demonstrated a densely packed and uniform cell population (Fig. 5B). Heat-inactivated EMAP II (at 1000 ng) was without effect on tumor histologic appearance (Fig. 5C). After administration of active EMAP II at 100 ng or 1000 ng twice daily for 12 days, a dose-dependent appearance of pyknotic bodies was observed (Fig. 5D-E), consistent with apoptosis, which appeared to parallel the course of capillaries. This was confirmed by assessing DNA fragmentation on sequential sections by end-labelling with digoxigenin-11-dUTP; compared with control tumors treated with vehicle alone, positive staining, characteristic of apoptosis, was observed in tumors treated with EMAP II (Fig. 5F-G). There was a dose-dependent increase in apoptotic areas present in the tumors with 100 and 1000 ng of EMAP II. Similar inhibition of tumor growth was observed when EMAP II was administered to mice with b16 melanomas.

These results led to an assessment of whether apparent necrosis in the perivascular areas of Meth A sarcomas, which produce EMAP II endogenously (as assessed by ELISA of meth A and tumor tissue), might be associated with apoptosis. DNA fragmentation was demonstrated by in situ nick translation in meth A, and appeared to parallel the vasculature in a pattern resembling that observed in LLC tumors in animals that received EMAP II. Pilot studies have

suggested that EMAP II was also present at these perivascular sites in the meth A tumor.

Effect of EMAP II on endothelium. EMAP II was initially isolated from Meth A tumors, due to their known thrombohemorrhage, resulting in spontaneously occurring areas of apparent necrosis/apoptosis (Old L., 1986). These data, along with examination of multiple LLC and B16 melanomas following treatment with EMAP II (in which apoptosis followed a perivascular pattern), suggested that EMAP II might modulate endothelial cell growth.

When confluent ECs were wounded and EMAP II was administered, cells at the wound edge failed to effectively migrate/proliferate and fill the wound area (Fig. 6B) compared to untreated controls (Fig. 6A). Staining with DAP-1, to visualize the chromatin, revealed the presence of apoptotic bodies, suggesting that EMAP II induced programmed cell death (Fig. 6D) versus their absence in controls (Fig. 6C). This effect was selective for rapidly growing ECs, as exposure of cultures approaching confluence to EMAP II had a small effect; there was a decrease in the mitotic rate and, at most, a 3-4 fold increase in apoptotic bodies (Fig. 6D) compared with untreated controls. EMAP II-induced apoptosis of rapidly growing ECs was further analyzed by electrophoresis for DNA fragmentation: characteristic ladder formation was observed in growing ECs exposed to EMAP II, whereas vascular smooth muscle cell DNA was unaffected.

As tumor tissue is also known for the presence of areas of local tissue hypoxia/hypoxemia (Olive P., 1992; Kalra R., 1994), whether EMAP II displays enhanced activity under oxygen deprivation was investigated. When ECs were exposed to hypoxia (pO_2 ≈14 torr) for 12 hrs, there was a decrease in mitotic rate (Fanburg B., 1987; Shreeniwas R., 1991) and a slight increase in apoptosis, which was magnified 50-fold in the presence of EMAP II.

Discussion

Formation of tumor vasculature is essential for growth and development of the neoplasm, but also provides an opportunity for therapy at the level of interrupting vascular integrity or the delivery of cytotoxic agents. Vasculature in tumors is known for its prothrombotic diathesis, increased permeability, exaggerated response to cytokines, and increased number of growing/migrating endothelial cells (Folkman, J., 1995; Old L., 1986; Asher A., 1987; Constantinidis I., 1989; Watanable N., 1988; Senger D., 1983). These properties, which distinguish vessels in the tumor bed from these in normal tissues, suggest parameters to be exploited in defining agents to selectively target tumor neovasculature.

Studies to identify EMAP II began with a characterization of mediators produced by Meth A tumor cells which perturbed properties of the endothelium (Kao J., 1992; Kao J., 1994; Nawroth P., 1988). Thus EMAP II was first studied based on its modulation of endothelial properties, such as induction of leukocyte adherence molecules and the procoagulant cofactor tissue factor. Further studies on mononuclear phagocytes and polymorphonuclear leukocytes confirmed its ability to induce cell migration and activation. These data suggested that EMAP II had properties of an inflammatory cytokine, at least based on in vitro findings. This was consistent with the capacity of EMAP II to enhance tumor thrombohemorrhage in response to TNF (Kao J., 1992; Kao J., 1994). However, the results of other in vivo experiments were not consistent with an important role for EMAP II as an inflammatory cytokine; in the footpad model, EMAP II induced only transient, mild swelling and leukocyte infiltration, and, following IV infusion, EMAP II elicited transient pulmonary leukostasis and expression of other cytokines (Interleukins 1 and 6, and tumor necrosis factor, TNF) (Kao J., 1992; Kao J., 1994). Furthermore, at the highest doses of EMAP II infused (10-50 μg/animal), there was no evidence

of severe toxicity and there were no fatalities (Kao J., 1994). This contrasts with the more potent effects of TNF under similar conditions (Beutler B., 1986). These data aroused suspicion that EMAP II might have properties distinct from proinflammatory cytokines. This supposition is supported by the finding reported herein that EMAP II had anti-angiogenic properties in vivo, in contrast to the angiogenic effects of TNF (Frater-Schroder M., 1987; Leibovich S., 1987).

In the current experiments EMAP II's anti-proliferative properties in vitro and antiangiogenic activity in vivo have been explored. Studies in the Matrigel model demonstrated reduction in neovascularization, consistent with endothelium comprising a cellular target of EMAP II. The diminished sized of tumors in the presence of EMAP II could result both from diminished neovascularization, as well as destruction of vessels already present in the tumor bed. The perivascular location of areas of apoptosis both in LLC, receiving exogenous EMAP II, and in Meth A tumors, producing EMAP II endogenously, suggests that vasculature was a target of the cytokine. The effect of EMAP II is less likely to be mediated by direct action on the tumor cells, as EMAP II does not impact adversely on tumor cell growth and viability in vitro. In contrast, experiments with cultured endothelium demonstrated induction of apoptosis of rapidly growing cultures, whereas there was a less pronounced effect on cultures approaching confluence. Although mitoses in just-confluent endothelium were markedly diminished, induction of programmed cell death was minimal, possibly to a cell cycle-dependence of EMAP II-induced cellular effects. As hypoxia is an important stimulus for angiogenesis, it was of interest to note that EMAP II had an exaggerated apoptotic effect in endothelial cultures subjected to oxygen deprivation. This was not observed in either smooth muscle cells or fibroblasts under similar hypoxic conditions. Data, showing high affinity endothelial binding sites for EMAP II, contrasted to the absence of such sites

on tumor cells, would be consistent with differential expression of EMAP II receptors on these cell types. Although the basis for this apparent specificity of EMAP II at the cellular level is at present unclear, this might reflect differences in receptor expression or post-receptor signalling. Such specificity is clearly critical for guiding future work directed at mechanisms underlying actions of EMAP II on the cellular and molecular levels.

RETINOPATHY

A hypoxia induced retinal neovascularization model has been well established by the "Association for Research in Vision and Ophthalmology Statement for the use of Animals in Ophthalmic and Vision Research," is followed. To produce retinal neovascularization, litters of 7 day old (postnatal day seven -P7) C57BL/6J mice with nursing mothers are exposed to 75% oxygen for 5 days and returned to room air at age P12 (room air will mimic hypoxia in the mouse). Animals receive IP vehicle (mouse serum albumin) - control, EMAP II 100-1000ng or heat inactivated EMAP II (1000ng) every twelve hours beginning on P7 and continuing until evaluation of retina. Mice of the same age kept in room air are used as controls. The eyes of the mice are evaluated on days P13-18 in room air for the development of retinopathy. This is accomplished by humane euthanasia of the mice, the infusion of a fluorescein-dextran solution and the use of fluorescence microscopy for the viewing of the eye vasculature. By assessing the amount of new vascularization, inhibition of retinal angiogenesis is demonstrated.

Example 2 Endothelial-monocyte Activating Polypeptide II, a Novel Anti-tumor Cytokine That Suppresses Primary and Metastatic Tumor Growth, and Induces Apoptosis in Growing Endothelial Cells

Neovascularization is essential for growth and spread of primary and metastatic tumors. From murine methylcholanthrene A-induced fibrosarcomas, well-known for their spontaneous vascular insufficiency, a novel cytokine has been identified and purified, Endothelial-Monocyte Activating Polypeptide (EMAP) II, that potently inhibits tumor growth in vivo, and appears to have anti-angiogenic activity in vivo and in vitro. Mice implanted with a matrix containing basic fibroblast growth factor showed an intense local angiogenic response which EMAP II blocked by 76% ($p<0.001$). Intraperitoneally administered recombinant EMAP II suppressed the growth of primary Lewis Lung Carcinomas, with a reduction in tumor volume of 65% compared with controls ($p<0.003$ by Mann-Whitney). In a lung metastasis model, EMAP II blocked outgrowth of Lewis lung carcinoma macrometastases; total surface metastases were suppressed by 65%, and of the 35% metastases present, about 80% of these were inhibited with maximum diameter <2 mm ($p<0.002$ compared with controls). In growing capillary endothelial cultures, EMAP II induced apoptosis in a time- and dose-dependent manner; an effect enhanced by concomitant hypoxia, whereas other cell types, such as Lewis Lung carcinoma cells, were unaffected. These data suggest that EMAP II is a tumor suppressive mediator with anti-angiogenic properties allowing it to target growing endothelium and limit establishment of neovasculature.

The following abbreviations are used herein below: meth A, methylcholanthrene A-induced fibrosarcoma; EMAP, Endothelial-Monocyte Activating Polypeptide; TNF, Tumor Necrosis Factor; EC, endothelial cell; SMC, smooth muscle cell; LPS, lipopolysaccharide; r, recombinant; BFGF, basis fibroblast growth factor; LLC, Lewis Lung Carcinoma; IV, intraperitoneal; IP, intraperitoneal; DAP-1, 6-diamidino-

2phenylindoledilactate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; VEGF, Vascular Endothelial Growth Factor.

Murine methylcholanthrene A-induced (meth A) fibrosarcomas, which exhibit spontaneous vascular insufficiency manifested by a heterogeneous pattern of thrombohemorrhage and central necrosis, as well as their failure to form metastatic lesions (Old, L., 1986; Old, L., 1961), provide an ideal starting point for isolation of tumor-derived mediators which perturb the vasculature (Clauss, M., 1990; Clauss, M., 1990; Kao, J., 1992; Kao, J., 1994). A novel cytokine-like molecule was purified, Endothelial-Monocyte Activating Polypeptide (EMAP) II, from meth A-conditioned medium based on its capacity to induce activation of endothelial cells and mononuclear phagocytes (Kao, J., 1992; Kao, J., 1994). This single chain polypeptide, devoid of a signal sequence, is initially synthesized as a ≈34 kDa intracellular precursor, which is processed to the mature ≈20 kDa form and released extracellularly by a yet to be identified pathway. EMAP II showed no significant homology to other known proteins, such as cytokines or growth factors. However, an aspartic acid residue is present in the P-1 position in both murine and human EMAP II, suggesting a cysteine protease in the Interleukin 1ß-converting enzyme family might be responsible for producing mature EMAP II from its pro-form. Our initial characterization of EMAP II suggested that its properties resembled those of proinflammatory mediators. For example, EMAP II induced endothelial release of von Willebrand factor, translocation of P-selectin to the cell surface, synthesis and expression of E-selectin and procoagulant tissue factor (Kao, J., 1992; Kao, J., 1994); these, and EMAP II-mediated activation of cultured monocytes, resulting in production of cytokines and stimulation of cell migration, suggested phlogogenic properties. However, EMAP II administered in vivo, locally or systemically, gave rise to, at most, mild and transient inflammation (Kao, J., 1994), suggesting that its effects

were quite different from those of tumor necrosis factor (TNF) or Interleukin 1 (Old, L., 1961; Sherry, B., 1988; Dinarello, C., 1993).

EMAP II has anti-angiogenic properties preventing blood vessel ingrowth in an experimental angiogenesis model, and suppressing the growth of primary and metastatic tumors without toxicity in normal organs. Consistent with this hypothesis, EMAP II appears to target growing endothelial cells; exposure of growing cultured capillary endothelium to EMAP II induces apoptosis, which is magnified by concomitant hypoxia. These data suggest that EMAP II is a polypeptide with anti-angiogenic properties which targets rapidly growing vascular beds, and suggests that, in addition to its effects on tumor neovessels, it may contribute to phases of normal development and wound repair in which cessation of blood vessel growth and tissue resorption are critical.

METHODS

Cell culture and in vitro assays. Bovine aortic and capillary endothelial cells (ECs) were isolated from calf aortae and adrenal, respectively, grown in culture and characterized, based on the presence of vonWillebrand factor and thrombomodulin, as described previously (Gerlach, H., 1989). Bovine vascular smooth muscle cells (SMCs) were prepared by additional scraping of the aortae following removal of the endothelium, and were characterized based on the presence of smooth muscle cell actin (Gown, A., 1985).

Lewis Lung Carcinoma (LLC) and B16(F10) melanoma cells, obtained from American Type Culture Collection (ATCC), were maintained in high glucose-defined Minimal Essential Medium (DMEM; Gibco) containing fetal bovine serum (10%). Meth A tumor cells (Center for Cancer Research, NY) were grown as described (Old, L., 1986). EMAP II-induced apoptosis was studied in subconfluent endothelial cultures (Gerlach, H., 1989). DNA fragmentation was quantified using a 5-bromodeoxyuridine (Brdu) incorporation kit from Boehringer-Mannheim according to the manufacturer's

instructions. In brief, cells were incubated for 12 hrs with BrdU, were plated for 24 hrs on 96-well plates, and were then treated with either vehicle (fetal bovine serum, 10%) alone or vehicle + rEMAP II, as indicated. After 12 or 24 hrs at 37°C, cells were lysed, centrifuged (250xg) for 10 min, and then the top 0.1 ml was aspirated and applied to an ELISA plate with pre-adsorbed anti-DNA antibody. Site of primary antibody binding were identified using peroxidase-conjugated anti-BrdU antibody. Where indicated, ECs were incubated with rEMAP II and/or exposed to hypoxia (pO_2 ≈14 torr) using a specially constructed controlled environment chamber, as described previously (Shreeniwas, R., 1991). After the incubation period, cells were fixed in paraformaldahyde (2.5%), rinsed with phosphate-buffered saline, incubated with 6-diamidino-2-phenylindoledilactate (DAP-1; final concentration, 1 ng/ml) and mounted with glycerol (10%). F-actin was visualized in cultured cells by incubation with rhodamine-conjugated phalloidin (Molecular Probes). Wounding of endothelial monolayers was performed using a 2-mm cork borer (Selden, S., 1981).

Preparation of recombinant murine EMAP II , and detection of EMAP II transcripts and antigen. Recombinant EMAP II was prepared from E. coli (host HMS174[DE3]) transformed with a plasmid containing the coding sequence for mature EMAP II, as described previously (Kao, J., 1994). Frozen (-80°C) E. coli cell paste was mixed 1:10 (w/v) with Tris-HCl (20 mM; pH 7.4) containing octyl-ß-glucoside (0.1%) and an homogeneous suspension was formed by agitation using a microfluidizer for 20 min (speed 60) at 4°C. Polyethylene imine at pH 7 was then added to the homogenate to a concentration of 0.25%, solids were removed by centrifugation (5000xg; 30 min), and the supernatant was retained. After filtration (0.2 μm), the sample was applied (3 mls sample/ml of gel) to Heparin Sepharose CL-4B (Pharmacia; 120 ml bed volume) equilibrated in Tris-HCl (20 mM; pH 7.4) containing octyl-ß-glucoside (0.1%), and the column was eluted with a linear ascending NaCl gradient.

Fractions were pooled on the basis of purity by silver stained SDS-PAGE, by immunoblotting with antibodies prepared to the N-terminus of mature EMAP II, and by biological activity measured in a tissue factor induction assay (Kao, J., 1994). The Heparin Sepharose pool was concentrated using an Amicon Stirred Cell (Amicon), the retentate was desalted into 3-(Morpholino)-propane-sulfonic acid (MOPS, 25 mM; pH 6.9), and was then applied to an SP Sepharose High Performance (Pharmacia) cation exchange column (55 ml bed volume). The column was eluted by application of a 0 to 0.5 M ascending linear salt gradient in MOPS, and EMAP II-containing fractions were adjusted to 2 M in $(NH_4)_2SO_4$, applied to a Phenyl Toyopearl 650 M (Tosohaas) column (90 ml bed volume), equilibrated in sodium phosphate (20 mM; pH 7) containing 1 M $(NH_4)_2SO_4$. The column was eluted with a descending salt gradient (2 to 0 M) in sodium phosphate (20 mM), and EMAP II in the Phenyl Toyopearl column eluate was concentrated to 3-5 mg/ml, and formulated into phosphate-buffered saline (PBS; pH 7.4) by buffer exchange on a Sephadex G25 column (as above). Lipopolysaccharide (LPS) was removed using filtration through a Posidyne filter (Pall Corp.), and LPS levels were estimated using the Endospecy chromogenic assay (limit of detection <10 pg/ml). Purified EMAP II was subjected to N-terminal sequence analysis, mass spectrometry and SDS-PAGE; the current material was found to be homogeneous according to these criteria. The phenyl-toyopearl column and Posidyne filtration steps appeared to remove certain toxic contaminant(s) associated with rEMAP II prepared by preparative electrophoresis in previous studies (Kao, J., 1994).

Antibody to rEMAP II was prepared by standard methods in rabbits (Vaitukatis, J., 1981) and was found to be monospecific, based on immunoblotting of plasma and cell extracts, and anti-EMAP II IgG blocked the activity of rEMAP II in cell culture assays (Kao, J., 1994). This antibody was used to develop an ELISA to detect EMAP II antigen by

the general protocol described previously (Kao, J., 1994).

PCR analysis for EMAP II transcripts employed RNA extracted from murine tissues (Balb/c mice) using the RNA Stat-60 kit (Teltest) according to the manufacturer's instructions, and reverse transcribed (1 μg) using Taq polymerase (Perkin-Elmer-Cetus). Primers were used for EMAP II (#1: GCATCGCGTCTGGATCTTCGAATT ; and, #2: GTATGTGGCCACACACTCAGCATT) and ß-actin (Gibco). Thermocycling parameters for the experiment shown in Fig. 7 were: 94°C for 30 sec; 55°C for 30 sec; and, 72°C for 30 sec for a total of 35 cycles. Samples were subjected to agarose gel (1%) electrophoresis and bands were visualized by ethidium bromide staining. Identity of amplicons was confirmed by Southern blotting with the appropriate cDNA probes.

Matrigel model. Matrigel (Kleinman, H., 1986; Passaniti, A., 1992) (Collaborative Research) containing either vehicle (1% BSA), rEMAP II (100 ng/ml) + vehicle; basic Fibroblast Growth Factor (bFGF; 100 ng/ml; Collaborative Research) + heparin (40 U/ml; Sigma) + vehicle; rEMAP II (100 ng/ml) + bFGF/heparin + vehicle; or heat-inactivated rEMAP II (100 ng/ml; alone or with bFGF/heparin) + vehicle was mixed at 4° C. Matrigel mixtures were injected subcutaneously into C57BL6/J mice (0.25 ml/site) at two sites per animal. The angiogenic response was analyzed at 7 and 14 days post-inoculation by routine histology and hemoglobin assay (Sigma).

Murine clearance studies. Clearance of EMAP II in mice was assessed using ^{125}I-labelled rEMAP II. rEMAP II was radioiodinated by the Bolton and Hunter method (3.2 mol of ester/mol of protein; 16), and the tracer was 99% precipitable in trichloroacetic acid (20%), migrated as a single band with Mr ≈20 kDa on SDS-PAGE, and had a specific radioactivity of ≈8000 cpm/ng. Balb/c mice received ^{125}I-rEMAP II (0.26 μg) either intravenously (IV) via the

tail vein or intraperitoneally (IP). Plasma samples were taken, and animals were sacrificed at 24 hours. Organs were then dried, weighed and radioactivity assessed. In addition, C57BL6/J mice bearing 14 day old subcutaneous B16 tumors received ^{125}I-rEMAP II (0.26 μg/animal; IP), and, 1 hour before sacrifice, were infused with ^{51}Cr-labelled microspheres (10 μ) in normal saline (the latter to monitor residual blood in the tissue). These studies were performed to define ^{125}I-rEMAP II plasma clearance, volume of distribution, and accumulation in tumor tissue. In each case, tissue associated radioactivity was determined on weighed samples either after drying (for total radioactivity), or following homogenization of tissue and trichloroacetic acid precipitation (20%). ^{125}I-rEMAP II in the tissue was corrected for residual blood based on the presence of ^{51}Cr-labelled microspheres. Plasma ^{125}I-rEMAP II concentration data were fit to a two-compartment open model using nonlinear regression by extended least squares analysis (Siphar, SIMED, Creteil, France). In order to assess the "goodness of fit," residual analysis (an examination of the standard deviation) was performed (Yamoaka, K., 1978).

Murine tumor models. *To test the effect of anti-EMAP II IgG on apoptosis in meth A tumors*, mice were subcutaneously injected with meth A cells and on day 9 started on a course of IP injections every third day of either nonimmune rabbit IgG (400 μg/dose) or rabbit anti-murine EMAP II IgG (200 or 400 μg/dose). This regimen of IgG administration was based on pilot studies in which ^{125}I-rabbit anti-EMAP II IgG infused into mice demonstrated a half-life of elimination of 29.4±2.67 hrs. Animals were sacrificed at day 14 and tissue was analyzed for evidence of apoptosis as described below.

For producing primary tumors to test the effects of EMAP II treatment, LLC and B16(F10) melanoma cells were rinsed with Hanks buffered saline solution, trypsinized, counted,

resuspended in phosphate-buffered saline, and injected subcutaneously into backs of C57BL6/J mice ($2x10^6$ cells/animal). On the third day following administration of tumor cells, the tumor was reproducibly measurable, and this tumor volume was taken for comparison with later measurements of that tumor. Animals then underwent IP injection every 12 hrs for 12 days of either vehicle alone (serum albumin, 1%), vehicle + rEMAP II (at 100 or 1000 ng), or vehicle + heat-inactivated rEMAP II (1000 ng). Tumor growth was assessed with calipers every third day (from days 3-15), and tumor volume was calculated according to the formula for a spherical segment (18), $V= \pi h(h^2+3a^2)/6$, where h= height of the segment, a= (length+width)/2, and V= volume (each tumor was compared with itself over multiple measurements and change in volume was noted). Tumor volume data were analyzed using the Kruskal-Wallis one way ANOVA and a Mann-Whitney mean rank test. Data is expressed as a dimensionless ratio of observed tumor volume divided by initial (day 3) tumor volume. Animals were sacrificed and tumors analyzed histologically at day 15.

For the metastatic tumor model (O'Reilly, M., 1994; Holmgren, L., 1995), C57BL6/J mice received LLC cells subcutaneously and were observed until tumor volume reached ≥1.5 cm^3. Animals then received rEMAP II (1000 ng/dose) in vehicle or vehicle alone IP every 12 hrs for 72 hrs prior to resection of the primary tumor. Following complete resection of the tumor (with no recurrence), mice were observed for an additional 15 days, during which time they received rEMAP II (1000 ng IP every 12 hrs) in vehicle or vehicle alone (same schedule). On day 15, lungs were injected intratracheally with India ink (15%) to visualize lung surface nodules, and tissue was fixed in Fekete's solution (70% alcohol; 5% glacial acetic acid; 3.7% formaldehyde). Surface metastatic lesions were counted by gross inspection of the tissue under 4X-magnification, and macrometastases were defined based on a smallest surface nodule diameter >2 mm.

Tissue analysis: histology, apoptosis, immunohistology. Histologic analysis was performed on formalin fixed, paraffin-embedded tissue, using hematoxylin and eosin staining. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was used to evaluate apoptosis; paraffin embedded tumor slices were deparaffinized and digoxigenin-11-dUTP was used to label fragmented DNA according to the Genius 1 kit (Amersham). In brief, tissue was treated with proteinase K (1 μg/ml), and incubated with digoxigenin-11d-UTP, klenow, and dNTP's overnight. Nitroblue tetrazolium and alkaline phosphatase were used to reveal the digoxigenin labelled DNA fragments. Where indicated, sections for the TUNEL assay were counterstained with eosin. For immunolocalization of EMAP II and thrombomodulin antigens, rabbit anti-rEMAP II IgG was employed (5 μg/ml) and rabbit anti-murine thrombomodulin IgG (2.5 μg/ml). Tissues, fixed as above, were incubated with primary antibody for 2 hr at 37°C or 1 hr at room temperature, respectively; sites of primary antibody binding were visualized using the ABC Elite kit (Vector), and revealed by reaction with 3,3'-diaminobenzidine.

RESULTS

Effect of anti-EMAP II IgG on meth A tumors. A heterogeneous picture of vascular insufficiency is commonly observed in meth A tumors shortly after the primary tumor becomes established (Old, L., 1986; Old, L., 1961). Prior to gross loss of tumor cell viability, pyknotic changes are evident in a perivascular distribution. At higher magnification, such pyknotic areas showed evidence of DNA fragmentation, based on TUNEL assay, and their general association with vasculature was confirmed by colocalization with the endothelial marker thrombomodulin (though regions of pyknosis/apoptosis extended beyond that in proximity to the blood vessel). Immunohistologic localization of EMAP II in meth A tumors showed it be associated with the vessel wall, possibly reflecting interaction with extracellular

matrix due to its heparin-binding properties. Furthermore, it appeared that expression of EMAP II protein in the tumor was evident by day 9 and continued to increase thereafter, which corresponds to the appearance of apoptotic changes in the tumor bed. Consistent with an association of EMAP II with meth A-associated apoptosis, the presence of such lesions was markedly diminished in mice treated with a high dose of anti-EMAP II IgG.

Distribution of EMAP II in normal mice. These data suggested that EMAP II could impact on tumor viability, which led to an examination of its sequestration in normal tissues. EMAP II transcripts were demonstrated in a range of organs (brain, liver, lung, spleen, heart, kidney, smooth muscle; Fig. 7), though their levels appeared to be quite low, requiring at least 35 cycles of PCR amplification to visualize the appropriate size amplicon. This impression was confirmed by Northern analysis, which showed a low intensity band at ≈1.1 kb in meth A cell RNA (corresponding to the size of the murine EMAP II mRNA; Kao, J., 1992), not observed in the normal organs. Expression of EMAP II transcripts was unaffected by infusion of lipopolysaccharide (LPS; 100 μg/animal) or induction of hind limb ischemia. ELISA for EMAP II antigen showed virtually undetectable levels in the above normal tissues (limit of detection <250 pg/ml) and no peak of EMAP II in the plasma after LPS administration or hind limb ischemia. These data indicated that EMAP II is expressed only at the lowest levels in normal mice, and that it is unlikely to be an early mediator of the host response to acute stimuli, such as LPS or ischemia. This clearly contrasts with the rapid production and significant roles for proinflammatory cytokines such as Interleukin 1 and TNF in the acute response to tissue injury (Old, L., 1961; Sherry, B., 1988; Dinarello, C., 1993).

Preparative scale purification of recombinant EMAP II. In order to further study the effects of EMAP II in vitro and in vivo, it was important to develop a preparative scale

purification procedure. Previously, material eluted from SDS-PAGE corresponding to Mr ≈20 kDa was employed. Although this material was highly purified, it was difficulat to scale-up such a method and the biologic properties of the resulting EMAP II were somewhat variable, probably due to differing degrees of denaturation/renaturaton during SDS-PAGE and gel elution. This led to development of an alternate purification strategy. Recombinant (r) EMAP II was expressed in E. coli, and purified by polyethylene imine precipitation followed by sequential application to Heparin Sepharose, SP Sepharose, and Phenyl Toyopearl. Posidyne filtration was then performed to remove LPS (levels were <10 pg at rEMAP II concentrations of 3-5 mg/ml). Details of chromatographic steps are described under Methods. The final formulated material was homogeneous on SDS-PAGE, migrating as a diffuse band at ≈21 kDa. Mass spectrometry gave a measured mass of 18,006 which is close to the expected mass of 17,970. N-terminal sequence analysis showed a single sequence with an 100% match between purified murine EMAP II and the published sequence (Kao, J., 1992; Kao, J., 1994).

Effect of EMAP II on bFGF-induced angiogenesis. To evaluate the ability of EMAP II to regulate blood vessel formation in response to known growth factors, bFGF and heparin were mixed with a gel of basement membrane proteins produced by Engelbreth-Holm-Swarm tumor cells (Matrigel) to serve as a model angiogenic stimulus (Kleinman, H., 1986; Passaniti, A., 1992). Subcutaneous Matrigel implants in C57BL6/J mice were evaluated 14 days after inoculation for vessel formation, cellular infiltration and hemoglobin content. Histologic analysis of the gel showed formation of vessels to be most pronounced and comparable in implants from animals treated with either bFGF/heparin + vehicle (albumin) or bFGF/heparin + heat-inactivated rEMAP II + vehicle; higher magnification confirmed the presence of neovessels in these implants. This induction of blood vessel formation is similar to that reported previously with bFGF in this model

(Passaniti, A., 1992). In contrast, in implants from animals treated with bFGF/heparin + active rEMAP II, there was marked reduction of vessel ingrowth; little-to-no vessel formation (n=40; this experiment was repeated seven times with similar results). Consistent with these histologic findings, there was a 76% reduction in hemoglobin content in corresponding implants containing bFGF/heparin + rEMAP II, compared to bFGF/heparin + vehicle or heat-inactivated EMAP II + bFGF/heparin +vehicle (see Figs. 3 A-E).

Plasma clearance and tissue deposition of infused rEMAP II. In order to perform in vivo studies with rEMAP II, its plasma clearance and tissue deposition were evaluated. Clearance studies were performed using either intravenously (IV) or intraperitoneally (IP) administered ^{125}I-rEMAP II (Fig. 4A-C). The fall in plasma concentration of ^{125}I-rEMAP II after IV injection fit best to a bi-exponential function (Yamoaka et al., 1978); the distribution and elimination half-lives were 0.47±0.17 and 103±5 min, respectively. Following IP injection, ^{125}I-EMAP II was detected in plasma after 1 min, and the maximum concentration was reached by 35±10 min. The resorption phase of rEMAP II handling in vivo was best described as a first-order process. The elimination phase following IP administration fit to a monoexponential decline, and the resorption and elimination half-lives were 50.1±0.1 and 102±6 min, respectively. Animals bearing B16 tumors were analyzed for tumor-associated radioactivity after receiving an IP injection of ^{125}I-rEMAP II; at 1, 6, and 12 hours radioactivity accumulated in the tumor was ≈7-fold greater than in the liver, and tumor selectivity was even more pronounced in other organs. Furthermore, the precipitability of the tracer in trichloroacetic acid (20%) was greater in the tumor compared with other tissues, consistent with a relative accumulation of apparently intact rEMAP II in tumor tissue.

Effect of rEMAP II on growth of primary and metastatic

tumors. Mice implanted subcutaneously with LLC cells developed tumors (the latter do not express detectable EMAP II protein), which were first measured when they achieved a volume of about 9-10 mm^3, ≈3 days following inoculation of cells. The volume of each tumor was then measured every third day, and compared with the initial volume of that tumor on day 3. Compared with tumor-bearing animals treated with vehicle alone or vehicle + heat-inactivated EMAP II, mice receiving active rEMAP II showed a striking reduction in tumor volume (Fig. 5A-G) Differences between tumor volume in control and EMAP II-treated animals were statistically significant using either the Kruskal-Wallis one way ANOVA analysis ($p<0.034$) or comparing control versus high dose rEMAP II by Mann-Whitney analysis ($p<0.003$). Histologic study of LLC tumors allowed to grow for 15 days and injected IP every 12 hrs with vehicle (albumin, 1%) demonstrated a densely packed and uniform cell population. Heat-inactivated rEMAP II (at 1000 ng/dose) was similar in appearance to the latter vehicle controls. After administration of rEMAP II at 1000 ng/dose twice daily for 12 days, areas of pyknosis were observed. At higher magnification, rEMAP II-induced areas of pyknosis had a general perivascular distribution, though pyknotic cells often extended beyond the vasculature. In Fig. %F-G, a site with several microvessels is visualized by staining for thrombomodulin, and evaluation of an adjacent section demonstrates DNA fragmentation using the TUNEL assay. There were no such apoptotic areas in control tumors treated with vehicle alone. There was a dose-dependent increase in apoptotic areas present in the tumors with 100 and 1000 ng of EMAP II. Similar inhibition of tumor growth was observed when EMAP II was administered to mice with primary B16 melanomas. Mice treated with rEMAP II were normally active, continued food/water consumption, and maintained their weights comparably to control mice.

As established metastatic foci require blood vessel ingrowth to expand beyond 1-2 mm (Fidler, I., 1994; Folkman, J.,

1989; Folkman, J., 1995; Murray, C., 1995), we reasoned that rEMAP II might suppress growth of metastatic lesions. The LLC model was employed by allowing primary tumors to grow to a volume of ≥1.5 cm^3, at which time metastases are present (but suppressed by the primary the primary tumor; O'Reilly, M., 1994; Holmgren, L., 1995). Then the primary lesion was resected (with no recurrence at the site of resection), and analysis of surface lung nodules was undertaken 15 days later. rEMAP II treatment was begun 72 hr prior to resection of the primary tumor and was continued through the end of the experiment (See Figs. 8A-E). Animals receiving rEMAP II (1000 ng IP every 12 hrs) showed significantly fewer and smaller surface nodules, compared with vehicle by gross inspection and histologic study. Consistent with these data, rEMAP II-treated animals demonstrated 65% suppression ($p<0.009$ by Mann Whitney) in outgrowth of the total number of surface metastases, compared with mice receiving vehicle alone (Fig. 8E). Of the 35% of metastases present in rEMAP II-treated animals, ≈80% of these metastases were inhibited, such that the maximum diameter was <2 mm (i.e., predominately micrometastases were present), compared with controls, ($p<0.002$ by student t-test; Fig. 8E; inset).

Effect of rEMAP II on endothelium. The data thus far demonstrated an association of EMAP II with spontaneous vascular insufficiency (meth A tumors) and with induction of apoptosis in tumors, the latter, at least in part in a perivascular distribution. These data suggested the possibility that tumor vasculature might be a target of EMAP II. To begin to assess whether rEMAP II selectively affects growing/migratory endothelium, confluent cultures were wounded and rEMAP II was added; cells at the wound edge failed to effectively migrate/proliferate and fill the wound area (Fig. 6B) compared to untreated controls (Fig. 6A). Staining with DAP-1, to visualize the chromatin, revealed the presence of apoptotic bodies localized to the wound edge (i.e., growing/migratory endothelium) in rEMAP II-treated

cultures, suggesting that rEMAP II induced programmed cell death only in this cell population, whereas confluent endothelium distal from the wound edge showed no significant effect of rEMAP II (Fig. 6D; arrows denote apoptotic bodies). Control cultures showed no such apoptotic areas (Fig. 6C). ELISA for DNA fragmentation was performed to more precisely delineate apoptotic effects of rEMAP II on endothelium: there was a dose-dependent increase in DNA fragmentation in cultured capillary endothelium, reaching 250% over that observed in controls within 24 hrs (Fig. 6E). As tumor tissue is also known for the presence of areas of local tissue hypoxia/hypoxemia (Olive, P., 1992; Kalra, R., 1994), it was assessed whether rEMAP II might display enhanced activity under oxygen deprivation. When cultured subconfluent endothelial cells were exposed to hypoxia (pO_2 ≈14 torr), DNA fragmentation was accelerated, reaching a level of 250% above that observed with vehicle alone within 12 hrs (rather than the 24 hrs required for an effect of this magnitude in normoxia). This was consistent with the accelerated appearance of apoptotic bodies by DAP-1 staining of hypoxic endothelial cultures exposed to rEMAP II. In contrast, cultured meth A fibrosarcoma, Lewis Lung carcinoma, and nontransformed vascular smooth muscle cells demonstrated no increase in DNA fragmentation after exposure to rEMAP II under the conditions above by ELISA (Fig. 6F) or DAP-1 staining.

DISCUSSION

Neovascularization is a critical regulator of the growth of both primary and metastatic neoplasms (Fidler, I., 1994; Folkman, J., 1989; Folkman, J., 1995; Murray, C., 1995). Earlier studies called attention to the role of angiogenic factors, such as vascular endothelial growth factor (VEGF; Plate, K., 1992; Warren, R., 1995; Kim, J., 1993), acidic fibroblast growth factor (Maciag, T., 1984), basic fibroblast growth factor (bFGF; Shing, Y., 1984), and angiogenin (Fett, J., 1985; King, T., 1991, Olson, K., 1994), in promoting tumor growth and establishing

metastases. For example, in a transgenic murine model, a switch in phenotype from pancreatic adenoma to malignancy was closely tied to expression of angiogenic mediators (Kandel, J., 1991), and antibody to VEGF inhibited growth of explanted human tumors in athymic mice (Warren, R., 1995; Kim, J., 1993). Similar inhibition of experimental tumor growth has also been observed with antibodies to angiogenin (Olson, K., 1994) and bFGF (Hori, A., 1991). Alternatively, recent work has identified endogenous peptides with anti-angiogenic activities, including angiostatin (O'Reilly, M., 1994), thrombospondin (Dameron, K., 1994) and glioma-derived angiogenesis inhibitory factor (Van Meir, E., 1994). They can inhibit tumor growth either at the primary tumor site (thrombospondin; Dameron, K., 1994) or at a site of distant metastases (angiostatin; O'Reilly, M., 1994; O'Reilly, M., 1996). Formation of the tumor vascular bed, as well as blood vessel formation in other situations, such as in ischemia, wound healing and atherosclerosis (Shweiki, D., 1992; Knighton, D., 1983; Kuwabara, K., 1995; Brogi, E., 1993), is presumably also controlled by the interaction of such positive and negative stimuli on endothelium in diverse vascular beds.

Carcinogen-induced murine meth A and similar tumors (Old, L., 1986; Old, L., 1961) are ideally suited to the analysis of host-tumor interactions because short-term vascular insufficiency (exaggerated by concomitant administration of an agent such as TNF), and longer-term immunologic mechanisms limit local tumor growth (Old, L., 1986; Old, L., 1961; Nawroth, P., 1988; Watanabe, N., 1988; Freudenberg, N., 1984; North, R., 1988). In fact, acute local (intratumor) administration of EMAP II to meth A tumors resulted in thrombohemorrhage in the tumor bed (Kao, J., 1994), a finding quite distinct from what we observed in the current study in which EMAP II was administered systemically at lower doses over longer times. The association of meth A-derived EMAP II with apoptosis in the tumor bed (the latter suppressed by anti-EMAP II IgG) and

immunolocalization of the polypeptide to vascular and perivascular areas of the tumor, suggested a role for this cytokine in vascular dysfunction associated with meth A tumors. Consistent with the ability of EMAP II to modulate vessel growth and/or integrity was the observation that neovessel formation into bFGF-containing implants was blocked by rEMAP II. In contrast to these results with rEMAP II, other cytokines such as transforming growth factor-ß or TNF-a have been found to induce vascular ingrowth in angiogenesis models (Leibovich, S., 1987; Fraker-Schroder, M., 1987; Madri, J., 1992).

In the LLC and B16 melanoma models, rEMAP II attenuated growth of primary tumors and resulted in a histologic picture of apoptotic tissue injury, at least in part in a perivascular distribution (similar to that seen with Meth A tumors), which progressed to nonviable tumor, probably as a result of severe ischemia . These data suggested that EMAP II was initially targeting the vasculature, leading to speculation that its tumor-suppressive effects might extend to a range of neoplasms. In support of this hypothesis, studies have shown rEMAP II to markedly attenuate growth of a human breast carcinoma line (MDA-MB-468) grown in nude mice and also to suppress C6 gliomas in rats. The observation that EMAP II diminished lung surface metastases, and, especially, macrometastases, is also consistent with the concept that neovasculature feeding the tumor, as well as in the tumor, are targets of EMAP II. It is notable that despite a prolonged course of rEMAP II treatment, ≈2 wks, no untoward effects on general health of the animals was observed, and pathologic analysis of normal organs revealed no lesions. This suggested that actions of EMAP II were localized, under these conditions, to the tumor. The data, however, do not rule out the possbility that EMAP II may have other effects on the tumor beyond that on the vasculature. For example, the action of EMAP II on endothelium or other elements in the tumor microenvironment might release diffusible mediators toxic for tumor cells,

thus causing tumor injury initially close to the vasculature, but then extending deeper into the tumor.

A salient feature of tumor vasculature, which distinguishes vessels in the tumor stroma from those in normal tissue, is the increased fraction of growing/migrating endothelial cells (Fidler, I., 1994; Folkman, J., 1989; Folkman, J., 1995). Studies in cell culture suggested a selective effect of rEMAP II on growing/migratory endothelium; cells at the leading edge of a wound in the monolayer failed to effectively fill the gap and cell proliferation was suppressed. The predominate affect appeared to be induction of apoptosis, especially in the actively dividing cell population. In contrast, postconfluent endothelium at a distance from the wound was not affected by rEMAP II. Furthermore, addition of the cytokine to cultures of growing tumor cells (LLC, B16 melanoma or meth A) showed no change in cell proliferation or induction of apoptosis, though rEMAP II suppressed these tumors in vivo. Enhanced EMAP II-induced apoptosis in hypoxic endothelial cultures provided further support for the relevance of our finding to tumor biology, as the presence of hypoxic areas in tumors is well-established (Olive, P., 1992; Kalra, R., 1994). On a cellular level, hypoxia could potentially sensitize endothelium to EMAP II by several mechanisms, including arrest of cells at the G1/S interface (Shreeniwas, R., 1991) or increased sensitivity to subsequent encounters with oxidizing stimuli. In support of the latter hypothesis, pilot studies suggest that EMAP II has an important effect on cellular redox status as addition of N-acetylcysteine blocks EMAP II-mediated endothelial apoptosis. Analysis of mechanisms through which EMAP II induces possible cellular oxidant stress, as well as elucidation of the cell surface receptor for EMAP II, will provide more definitive answers to questions concerning the specificity and selectivity of its cellular effects.

The striking feature of the in vivo studies is the

suppressive effect of rEMAP II on tumors without, apparently, an adverse affect on the function of normal organs. This may be due to EMAP II's effect on the endothelium; EMAP II could perturb endothelium in vivo not only by direct effects on endothelial apoptosis, but also by other means. For example, EMAP II-mediated induction of endothelial tissue factor could trigger local activation of clotting in the tumor bed, thereby diminishing blood flow and enlarging the volume of tumor at risk for ischemia. EMAP II might also modulate the expression of other mediators which control the local angiogenic balance, including enhanced activity of pathways regulating production of angiostatic peptides, such as angiostatin or thrombospondin, and/or might suppress expression of pro-angiogenic factors in the tumor bed. Furthermore, EMAP II might elicit endothelial production of mediators which directly impair tumor cell viability (as mentioned above). Though there are many mechanistic, physiologic and practical questions to be explored in future studies (Will EMAP II affect well-established vessels in human tumors which grow over much longer times than the accelerated murine models? Will an optimal anti-tumor regimen of EMAP II induce tumor regression or will it just be static? etc.), the data support the potential of EMAP II, a cytokine with apparent anti-angiogenic properties, to suppress primary and metastatic tumor growth, and to induce apoptosis in the tumor without apparent adverse affects on normal organs.

Example 3:

ENDOTHELIAL-MONOCYTE ACTIVATING POLYPEPTIDE II SUPPRESSES GROWTH OF C6 GLIOMAS BY TARGETING THE VASCULATURE

Endothelial-Monocyte Activating Polypeptide (EMAP) II is a novel mediator initially purified from methylcholanthrene A-induced fibrosarcomas, well-known for spontaneous vascular insufficiency and thrombohemorrhage. Testing the effect of EMAP II on C6 gliomas which elicit a characteristic angiogenic response, largely due to expression of Vascular Endothelial Growth Factor (VEGF) was therefore carried out.

Recombinant (r) EMAP II suppressed intracranial growth of C6 glioma cells implanted in rat frontal lobes by 65% ($p<0.003$), with residual tumor volume composed predominately of apoptotic cells (≈80%). Similarly, rEMAP II had a striking effect on C6 gliomas grown subcutaneously in nude mice, causing a six-fold decrease in tumor volume, without evidence of systemic toxicity. rEMAP II blocked the angiogenic response to locally administered VEGF, demonstrating a direct effect of EMAP II on VEGF-driven vascular ingrowth. Ultrastructural study of tumor vasculature from animals treated with rEMAP II showed intravascular accumulation of platelets and fibrin, as well as findings consistent with apoptosis of the endothelium. Consistent with the ability of EMAP II to target the vasculature, rEMAP II induced apoptosis and bound specifically (K_d ≈xx nM) to growing cultures of human umbilical vein endothelial cells, whereas it had no affect and displayed no specific binding to C6 glioma cells. These studies demonstrate that EMAP II has anti-tumor activity on C6 gliomas, at least in part, through its affect on the vasculature, and suggest its possible application to a range of solid tumors.

The following abbreviation are used herein below: VEGF, Vascular Endothelial Growth Factor; EMAP; Endothelial Monocyte Activating Polypeptide II; r, recombinant; IP, Intraperitoneal; IT, Intratumoral; TUNEL, Deoxynucleotidyl Transferase-mediated DUTP-biotin nick end labelling.

Vascularization of solid tumors is critical for their growth beyond a small collection of neoplastic cells (Fidler, I., 1994; Folkman, J., 1989; Folkman, J., 1995). In the central nervous system, in which vasculature is insulated from the neuronal compartment by the blood-brain barrier, effective mechanisms for induction of neovasculature have evolved to support tumor growth. Glioblastoma, the most frequently occurring intracranial neoplasm, displays characteristic vascularization with evidence of endothelial proliferation

and a complex vascular network, thereby providing an especially relevant example of ongoing angiogenesis (San Galli, F., 1989; Plate, K., 1992; Wesseling, P., 1994; Plate, K., 1995). Although induction of vascular ingrowth by glioblastomas is likely to involve multiple mediators, studies with patient-derived glioblastoma multiforme and rat gliomas have emphasized the contribution of Vascular Endothelial Growth Factor (VEGF) (Plate, K., 1992; Shweiki, D. 1992; Weindel, K., 1994; Plate, K., 1994; Samoto, K., 1994). The secreted isoform of VEGF (residues 1-165) is produced by glioblastoma/glioma at the tumor margin, especially at sites of local necrosis (and presumably, hypoxia), enhancing neovessel formation by attracting endothelium which has been shown to selectively express the VEGF receptor Flk-1 (Plate, K., 1992; Shweiki, D. 1992; Weindel, K., 1994; Plate, K., 1994; Samoto, K., 1995). Direct evidence of a role for VEGF in glioma growth derives from experiments demonstrating that antibodies to VEGF (Kim, K., 1993), a dominant negative mutant of Flk-1/VEGF (Millauer, B., 1994), and VEGF antisense introduced into gliomas suppresses the tumors (Saleh, M., 1996).

VEGF has emerged as an angiogenic factor involved in physiologic and pathophysiologic vascular responses (Houck, K., 1991; Keck, P., 1989). This polypeptide was initially characterized based on its ability to increase vascular permeability when injected subcutaneously into guinea pigs (Keck, P., 1989). In this context, VEGF has been show to have other properties associated with inflammatory mediators in vitro, including induction of the procoagulant tissue factor on endothelial cells and mononuclear phagocytes (Clauss, M., 1990). Although the relevance of these findings to the biology of VEGF in vivo has not been clarified, it has been speculated that this could account for pathologic findings in the vasculature of gliomas, including evidence of vascular leakage and local thrombi. The role of VEGF as a central angiogenic mediator has been demonstrated more directly. Deletion of the VEGF gene

results in an embryonic lethal, with failure of vasculogenesis (Harpal, K., 1996). In pathophysiologic situations, VEGF has been implicated in neovascularization associated with diabetic retinopathy, ischemic events, and tumor growth (Shweiki, D. 1992; Weindel, K., 1994; Plate, K., 1994; Samoto, K., 1994; Miller, J., 1994; Aiello, L., 1994).

Endothelial-Monocyte Activating Polypeptide (EMAP) II is a novel mediator initially identified in meth A tumors, well-known for their spontaneous vascular insufficiency (Kao, J., 1992; Kao, J., 1994). Acute administration of EMAP II directly into tumors elicited thrombohemorrhage and sensitized tumor vasculature to subsequent systemic infusion of tumor necrosis factor. Treatment of mice bearing Lewis Lung Carcinomas or B16 melanomas with low concentrations of EMAP II administered systemically for several weeks resulted in tumor regression and a pathologic picture of patchy apoptosis apparently radiating from tumor vasculature (Schwarz, M., 1995). These findings, along with the ability of EMAP II to inhibit vascular ingrowth elicited by implants impregnated with basic fibroblast growth factor and its lack of a direct cytotoxic/cytostatic effect on tumors cells, suggested that EMAP II might target tumor vasculature. The goal of the studies was to determine if EMAP II could antagonize the angiogenic effects of glioma-derived angiogenic factors, especially VEGF, thereby implying its potential to block pathologic vascular ingrowth. The results herein indicate that systemically administered EMAP II blocks neovessel formation in response to VEGF in a Matrigel model, and that it potently suppresses growth of C6 gliomas. Ultrastructural studies revealed that EMAP II induced early changes in the vasculature including intravascular fibrin formation, deposition of platelets, and findings consistent with endothelial apoptosis. This was consistent with the results of in vitro functional and binding studies which indicated that endothelial cells, rather than C6 glioma cells, were the target of EMAP II.

MATERIALS AND METHODS

Cell culture and preparation of recombinant (r) EMAP II. C6 glioma cells (Benda, P., 1971) were obtained from ATCC and were grown in Dulbecco's Modified Eagle Medium containing fetal bovine serum (10%; Gemini, Gibco, Grand Island NY). Mouse brain endothelial cells were characterized and grown as described (Gumkowski, F., 1987). Human umbilical vein endothelial cells were grown and characterized as described (Kao, J., 1992). DNA fragmentation was evaluated by agarose gel electrophoresis (xBorczyca et al., 1993- ask dave p.). Radioligand binding studies employed ^{125}I-rEMAP II and cultured C6 glioma or endothelial cells. rEMAP II was radiolabelled by the Bolton and Hunter method (Bolton, A., 1973); the tracer was >95% precipitable in trichloroacetic acid (20%) and migrated as a single band with Mr ≈20 kDa on SDS-PAGE. rEMAP II was prepared from E. coli transformed with a plasmid containing the coding sequece for mature murine EMAP II, and was purified by a modification of our previous procedure (Kao, J., 1994) using sequential chromatography on Heparin Sepharose, SP Sepharose, and Phenyl Toyopearl chromatography, followed by filtration through a Posidyne filter to remove lipopoysaccharide. The final material was migrated as a single band on SDS-PAGE (reduced and nonreduced) with Mr ≈20 kDa, had a single N-terminal sequence by mass spectrometry, and had a lipopolysaccharide content of <10 pg/ml (at a protein concentration of 3-5 mg/ml based on the Endospecy chromogenic assay; Seigaku). The protocol for binding included washing cultured endothelium ($2x10^4$ cells/well) in Hanks' balanced salt solution, and then adding Minimal Essential Medium containing fetal bovine serum (10%) at 4°C containing ^{125}I-rEMAP II alone or in the presence of an 100-fold molar excess of unlabelled rEMAP II. Wells were incubated for 2 hrs at 4°C, unbound material was removed by six rapid washes (for a total of 6 sec/well) with phosphate-buffered saline, and cell-associated radioactivity was eluted with phosphate-buffered saline containing Nonidet

P-40 (1%). Specific binding (total binding observed in wells incubated with ^{125}I-rEMAP II alone minus nonspecific binding observed in wells incubated with ^{125}I-rEMAP II + excess unlabelled rEMAP II) is shown in the figures, and was analyzed by the method of Klotz and Hunston (Klotz, I., 1984).

Matrigel model. Matrigel (Collaborative Research)(Kleinman, H., 1986; Passaniti, A., 1992) containing either vehicle (1% bovine serum albumin), VEGF (100 ng/ml; Collaborative Research) + vehicle, or heat-inactivated VEGF (15 min at 100°C) + vehicle (mouse serum albumin, 1 mg/ml) was mixed at 4°C. Matrigel mixtures (0.25 ml/site; two sites per animal) were injected subcutaneously into C57BL6/J mice (0.25 ml/site) at two sites per animal. Animals were treated with rEMAP II (1 μg/12 hrs; IP) starting on the day that Matrigel was implanted, and for the next 14 days, at which time Matrigel was harvested. Vascular ingrowth was analyzed by routine histology and hemoglobin assay (Sigma). Each experiment employed five animals per group.

Tumor models. All protocols were approved by the Columbia IACUC. C6 glioma cells were implanted into the frontal lobe of Wistar rats (250-300 grams; Charles River) by a modification of methods described in the literature (San-Galli, F., 1989; Bernstein, J., 1990). In brief, following anesthesia with ketamine (90 mg/kg; IP Parke-Davis, NJ) and xylazine (10 mg/kg; IP; American Health Co., Kansas), animals were placed in the sterotactic head frame, a 2-3 mm hole was made in the skull, the dura was opened, and the stereotactic apparatus was used to place a rod (23 gauge stainless needle) 4.5 mm into the deep white matter of the right frontal lobe (coordinates for the burr hole were 1 mm anterior to the coronal suture and 3 mm lateral to the sagittal suture). Rats were allowed to recover for 48 hrs, and then, following anesthesia, C6 glioma cells were administered ($4x10^4$ cells in 5 μl of Hanks balanced salt solution) at an infusion rate of 1 μl/min. After an

additional 10 days, tumor-bearing rats were divided eight different treatment groups (N=9 per group for a total of 72 animals/experiment): (1) rEMAP II (100 ng) administered intratumorally (IT) every 48 hrs (40 μl over 133 min) in vehicle (rat serum albumin, 1 mg/ml) + rEMAP II (10 μg) administered intraperitoneally (IP) every 12 hrs in vehicle; (2) rEMAP II (100 ng) IT in vehicle; (3) rEMAP II (10 ng) IT in vehicle + rEMAP II (1 μg) IP in vehicle; (4) rEMAP II (10 μg) IP in vehicle; (6) vehicle IT every 48 hrs + vehicle IP every 12 hrs; (7) vehicle IP every 12 hrs; (8) vehicle IT every 48 hrs; and, (9) heat-inactivated rEMAP II (100 ng) IT in vehicle every 48 hrs + heat-inactivated rEMAP II (10 μg) IP in vehicle every 12 hrs. Intratumoral administration involved positive pressure microinfusion through the implanted rod at a volume of 40 μl infused over 133 min. Once the treatment regimen including rEMAP II was begun, it was continued for a total of either 7 or 14 days. There were 8 eight animals in each treatment group. At the end of the experiment, animals were sacrificed by humane euthanasia, the cranium was opened, the brain removed, incubated in formalin (4%) at 4°C for 72 hrs, and placed in a brain matrix to make serial 1 mm coronal slices. The latter were paraffin-embedded, sectioned coronally (4 μm), and underwent routine histology (hematoxylin/eosin) and TUNEL assay (see below). Tumor volume was calculated according to the formula for a spherical segment (see below; Weast R., 1966) based on the largest cross-sectional tumor diameter, and serial images were evaluated by NIH image.

Tumors were grown subcutaneously in nude mice (Taconic) by administering C6 glioma cells ($2x10^6$ suspended in phosphate-buffered saline; Kim, K., 1993; Millauer, B., 1994), and on the third day animals were divided into thre groups (N=10-15/group): (1) vehicle (mouse serum albumin, 1%) given IP every 12 hrs; (2) rEMAP II (1 μg) in vehicle given IP every 12 hrs; and, Folkman, J., 1995) heat-inactivated rEMAP II (1 μg) in vehicle given IP every 12 hrs. Initial tumor size (i.e., prior to treatment on day

3) in each of the groups was 12-14 mm^3. This treatment regimen was continued for up to 31 days. Tumor dimensions were measured every fourth day, and these data were used to calculate tumor volume according to the formula for a spherical segment (Weast R., 1966), $V= \pi h(h^2+3a^2)/6$, where h= height of the segment, a= (length+width)/2, and V= volume (each tumor was compared with itself over multiple measurements and change in volume was noted). Tumor volume data were analyzed using the Kruskal-Wallis one way ANOVA and a Mann-Whitney mean rank test. Data is expressed as a dimensionless ratio of observed tumor volume divided by initial (day 3) tumor volume. Animals were sacrificed and tumors analyzed histologically at the indicated times.

Histologic studies were performed on formalin-fixed, paraffin-embedded tissue, using hematoxylin and eosin staining. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) assay was used to evaluate apoptosis; paraffn embedded tumor slices were deparaffinized and digoxigenin-11-dUTP was used to label fragmented DNA according to the Genius 1 kit (Amersham). In brief, tissue was treated with proteinase K (1 μg/ml), and incubated with digoxigenin-11-dUTP, klenow, and dNTPs overnight. Nitroblue tetrazolium and alkaline phosphatase were used to reveal the digoxigenin labelled DNA fragments.

Ultrastructural studies. Glioblastomas were raised subcutaneously in nude mice, and tumors were removed and processed for electron microscopy on days 3, 6 and 9 (Roberts, W., 1995). In brief, tumor specimens were cut into small pieces and immediately fixed by immersion in glutaraldehyde (1.5%) in sodium cacodylate-HCl (0.1 M; pH 7.4) with sucrose (5%) for 1 hr. Following washes in cacodylate buffer (0.1 M) with sucorse (7.5%), specimens were post-fixed in cacodylate-buffered (pH 7.4) osmium tetroxide (1%) for 1 hr, en-bloc staine with uranyl acetate overnight, dehydrated, embedded in EPON 812, and cured for

18-24 hrs at 60°C. Thin (50-55 nm) sections were cut (Reichert-Jung Ultracut E, Austria) piked up on copper grids, stained with uranyl acetate and lead citrate before examination and photographing on a Phillips CM10 electron microscope at 80 kV.

RESULTS

Effect of rEMAP II on growth of C6 gliomas in vivo. C6 glioma cells were implanted stereotactically in the right frontal lobe of Wistar rats. This model was selected based on previous studies demonstrating that histologic features of these tumors closely resemble findings in tumors of patients (San-Galli, F., 1989; Bernstein, J., 1990). Tumor growth occurred steadily up to about 28 days, when death resulted from increased intracranial pressure. For this reason, experiments were terminated at day 24; there were no fatalities at this time. To assess the effect of rEMAP II on growth of C6 gliomas, three different treatment regimens were employed: intra-tumoral (IT) administration via indwelling cannula, intraperitoneal (IP) administration, and both IT + IP (IT/IP) administration. IT treatment, via indwelling cannula according to our protocol, has been shown to effectively deliver therapeutic agents within the central nervous system without elevating intracranial pressure. Animals receiving rEMAP II by the IT/IP routes showed the greatest suppression of tumor growth; at a dose of 100 ng (IT)/10 μg (IP), tumor volume was diminished by ≈3-fold compared with tumors treated according to the same protocol with vehicle alone (IT/IP, IP or IT) ($P<0.002$). The effect of rEMAP II was dose-dependent, being diminished at 10 ng (IT)/1 μg (IP), in which case tumor volume was diminished, but did not achieve statistical significance compared with controls. Rats treated with rEMAP II (10 μg) by the IP route alone showed little change in tumor growth, whereas rEMAP II (100 ng) given only IT had a striking effect, though it was somewhat less effective than combined IP/IT administration. Controls in which heat-inactivated rEMAP II replaced active rEMAP II demonstrated no reduction in tumor

size compared with vehicle-treated controls. Histologic analysis of control tumors (either no treatment or IP/IT vehicle alone) showed a relatively homogeneous central region of the tumor (Fig. 9B-9C) with areas of palisading tumor cells and necrosis at the periphery. In the central region of the tumor from control animals, where necrosis was minimal, there was also little evidence of DNA fragmentation, based on the TUNEL assay (Fig.9D-9E). In contrast, tumors from animals receiving rEMAP II (IP/IT) demonstrated marked inhomogeneity with pyknotic areas (Fig. 9C-9D) in which DNA fragmentation was ubiquitous. Quantitative analysis using NIH image indicated that C6 gliomas treated with rEMAP II (IP/IT) had ≈80% of the residual volume accounted for by apoptotic cells. As the treatment regimen with rEMAP II was modified from 100 ng (IP)/10 μg (IT) to lower concentrations, either 10 ng (IT)/1 μg (IP), or 1 μg (IP) or 100 ng (IT) alone, resulting in less effective suppression of tumor growth, histologic evidence of pyknosis and apoptosis decreased. In contrast to the effect of rEMAP II (IP/IT) on tumors, there was no evidence of toxicity even after 14 days of treatment. There were no deaths in the treatment group; animals displayed normal activity, behavior (no seizures or other untoward events were observed), and food intake.

To further examine the effect of rEMAP II on C6 gliomas, experiments were performed with tumor cells inoculated subcutaneously in nude mice. Tumor cells were implanted into immunocompromised mice, and growth was allowed to occur for three days, at which time palpable tumors were reproducibly evident (approximate volume prior to treatment was 12-14 mm^3, in each group). rEMAP II was then administered starting on day 3, and tumor volume was measured every four days thereafter; data are reported at each time point as fold-change in tumor volume (a dimensionless ratio comparing tumor volume on the indicated day with that on day 3); this method allowed a comparison of each animal with itself. Tumors in rEMAP II-treated animals

displayed ≈6-fold reduction in volume compared with tumors in mice receiving vehicle alone by day 31 (Fig. 9E). Histologic appearance of rEMAP II-treated C6 gliomas showed small tumors with evidence of pyknotic changes and apoptosis throughout the lesions, compared with larger tumors in vehicle-treated controls which displayed homogeneous central areas and apoptotic/necrotic changes limited to the periphery.

VEGF-mediated vascular ingrowth into Matrigel implants: effect of rEMAP II. Matrigel is a complex mixture of basement membrane proteins, as well as other cell products, from Engelbreth-Holm-Swarm (EHS) tumor cells (Kleinman, H., 1986; Passaniti, A., 1992). Addition of an exogenous growth factor, such as basic fibroblast growth factor, to Matrigel has been shown to provide a model for assessment of vessel ingrowth (Kleinman, H., 1986; Passaniti, A., 1992). This model was employed by mixing Matrigel with recombinant human VEGF and subcutaneously implanting the mixture into mice. Animals were then treated with either rEMAP II (1 μg) in vehicle, heat-inactivated rEMAP II (1 μg) in vehicle or vehicle alone every 12 hrs for 14 days. Implants containing VEGF in animals not receiving active rEMAP II (i.e., vehicle controls or heat-inactivated rEMAP II) showed numerous vascular structures (Figs. 10A-B show the results with vehicle alone administered IP and VEGF in the Matrigel implant). Vascular ingrowth was markedly inhibited in the presence of active rEMAP II administered IP (Fig. 10C-D). Consistent with these results, hemoglobin assays on Matrigel implants, as an estimate of vascularization, showed reduced hemoglobin content in samples from animals treated with rEMAP II (Fig. 10E). Controls in which VEGF was heat-inactivated demonstrated suppression of the angiogenic response, whereas heat-inactivation of rEMAP II in implants containing active VEGF did not diminish neovessel formation. These data suggested that the vasculature was a target of EMAP II, and led us to re-assess normal organs in animals treated with rEMAP II. No change was observed in micro- or

macro-vessels from the organs, such as heart, lung, spleen, or kidney), suggesting that the effects of EMAP II were focussed on the tumor bed.

Ultrastructural properties of tumor vasculature: effect of rEMAP II. Tumors harvested after 3, 6 or 9 days of EMAP II treatment were noticeably different from controls. Macroscopically, reddish pinpoint areas were observed, presumably the result of red blood cell stasis, extravsation and thrombus formation. This impression was confirmed by microscopic studies showing platelet thrombi and red cell stasis, especiallly in large (40 μm dimabeter) venular vessels. Consistent with the presence of fibrin, ultrastructural studies showed a 21 nm periodicity of the fibrin strands. Vasculature in both control and EMAP II-treated tumors displayed attentuated endothelium, often with fenestrations and open interendothelial junctions (Roberts, W., 1995). However, platelet thrombi and fibrin clots were noticed only in EMAP II-treated tumors.

Interaction of rEMAP II with cultured endothelial cells. These data suggested that EMAP II selectively interacted with vascular elements in the tumor bed. In support of this concept, human umbilical vein endothelial cells exposed to rEMAP II showed increased DNA fragmentation by agarose gel electrophoresis, compared with untreated controls (Fig. 11A). Also, ^{125}I-rEMAP II bound to cultured endothelium in a dose-dependent manner, demonstrating K_d = 1.9 nM (Fig. 11B). In contrast to these results with endothelial cells, rEMAP II had no affect on C6 glioma cells with respect to apoptosis (or changes in cell number). Also, there was no specific binding of ^{125}I-rEMAP II to cultured C6 glioma cells. These data suggest the existence of functional rEMAP II binding sites/receptors on endothelium.

DISCUSSION

Recent studies on glioblastoma multiforme have focussed attention on tumor vasculature as a model system for

analysis of angiogenic mechanisms and for examination of potential future therapeutic modalities (San-Galli, F., 1989; Plate, K., 1992; Wesseling, P., 1994; Plate, K., 1995; Shweiki, D., 1992; Weindel, K., 1994; Plate, K., 1994; Samoto, K., 1995; Kim, K., 1993; Millauer, B., 1994; Saleh, M., 1996). Areas of necrosis at the tumor margin, along with the presence of palisading tumor cells which express VEGF, have emphasized the possibility that induction of vascular ingrowth is a limiting factor for growth of the neoplasm, and that VEGF may have a central role in tumor survival. This concept is supported by the results of studies demonstrating that antagonism of VEGF with specific antibodies, at the level of the endothelial receptor Flk-1, or with antisense technology supresses tumor formation (Kim, K., 1993; Millauer, B., 1994; Saleh, M., 1996). The current data add to and extend the concept that a mediator with properties which impact negatively on vascular integrity might inhibit growth of glioblastomas. Further support for the value of such an approach to therapy of glioblastomas is also provided by recent studies outlining anti-angiogenic strategies for glioma therapy (Johnson, J., 1996).

EMAP II has several properties which are consistent with the hypothesis that it specifically affects tumor vasculature. First, experiments in tissue culture demonstrate specific, high affinity binding to endothelium, but not to C6 glioma cells. Second, EMAP II induction of endothelial apoptosis appears limited to growing endothelium, a situation particularly relevant to tumor vasculature (Fidler, I., 1994; Folkman, J., 1989; Folkman, J., 1995; O'Reilly, M., 1994; Holmgren, L., 1995). These observations in tissue culture provide support for the results of our in vivo studies in which animals receiving EMAP II demonstrated suppression of tumor growth, but did not display toxic effects in the vascular bed of normal organs. Third, EMAP II administered systemically antagonizes VEGF-induced neovessel formation in the Matrigl model, whereas no untoward reactions occurred in established vasculature

outside the implant. In addition to antagonism of VEGF, EMAP II may provide a broader spectrum of activities which impact negatively on tumor survival in the host, including inhibition of other angiogenic activities in the tumor, such as basic fibroblast growth factor (Stan, A., 1995). Further, EMAP II induction of endothelial tissue factor (Kao, J., 1992; Kao, J., 1994) potentially underlies vascular fibrin formation, ultimately leading to occlusive thrombosis and cessation of blood flow. Ultrastructural analysis of tumor vasculature from EMAP II-treated animals confirmed the presence of both fibrin and findings consistent with apoptosis of the endothelium.

Although it is difficult to dissect with certainty the exact mechanism through which EMAP II exerts its affects on tumors from the pathologic picture in treated tumors bed of tumors treated with EMAP II, a mechanism other than direct tumor cell cytotoxicity seems likely. If this proves to be true, the most effective therapy might be to combine EMAP II with agents directly targetting neoplastic cells, such as cytotoxic agents or anti-sense to Insulin-like Growth Factor, the latter having been shown to suppress glioma growth (Resnicoff, M., 1994). One possible insight into the complexity of EMAP II's actions in the tumor bed is suggested by the greatly enhanced anti-tumor effect following intratumor injection. The observation herein that EMAP II administered only via the systemic route (IP) had little effect on tumor growth suggests that adequate access to the central nervous system was not possible following systemic delivery. Delivery of anti-tumoral compounds to brain tumors is compromised by systemic methods which limit effective drug concentrations due to limitations imposed by the blood brain barrier (as with EMAP II which is a polypeptide of 22 kDa) and/or systemic degradation/clearance of compounds (Tomita, T., 1991). These limitations can be safely circumvented by local delivery methods utilizing high flow positive pressure microinfusion directly into the tumor through the interstitial spaces by bulk flow. The drug

concentration drops off rapidly at the edge of the infusion front (i.e., high local concentrations of drug can be achieved) and adjustments can be made in the rate and volume of the infusion to achieve desired constant tissue concentrations. Bulk flow is less dependent on the physical characteristics of the substance being infused and is therefore uniquely suitable for complex macromolecues which do not diffuse well and are difficult to deliver systemically. Efficacy of this delivery system, without complications, using EMAP II demonstrates the potential for clinical development of other novel compound for anti-tumoral therapeutics that would otherwise be impractical if delivered systemically.

Delivering therapeutic agents to the central nervous system poses special difficulties because of the presence of the blood-brain barrier. Although neovasculature in glioblastomas displays histologic features which suggest increased permeability, the likelihood of gross breakdown of the blood-brain barrier is low. The ability to deliver EMAP II via an indwelling catheter in the region of the tumor, whithout raising intracranial pressure or causing other untoward effects in the central nervous system, introduces the therapeutic agent in proximity to glioma cells, the primary source of angiogenic stimuli. Combined therapy, both systemic and local, was most efficacious, possibly by maximizing delivery of EMAP II to both tumor vessels, interstitium and the neoplastic cells. These data emphasize a role for local delivery of therapeutic agents to intracranial tumors, and suggest the importance of developing and testing such systems which can be ultimately applied to clinical practice.

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What is claimed is:

1. A method of treating a tumor in a subject, comprising administering to the subject an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to treat the tumor, wherein the endothelial monocyte activating polypeptide II is administered subcutaneously, intraperitoneally, or intravenously.

2. The method of claim 1, wherein the tumor is a carcinoma.

3. The method of claim 1, wherein the subject is a mouse.

4. The method of claim 1, wherein the agent is administered intraperitoneally.

5. The method of claim 1, wherein the agent is administered in at least twenty doses.

6. The method of claim 5, wherein the agent is administered in about twenty-four doses.

7. The method of claim 1, wherein the agent is administered over a period of at least ten days.

8. The method of claim 7, wherein the agent is administered over a period of about twelve days.

9. The method of claim 1, wherein the frequency of administration is at least about one dose every twelve hours.

10. The method of claim 1, wherein the effective amount is from about 2.4 micrograms to about 24 micrograms.

11. The method of claim 1, wherein the effective amount is from about 100 nanograms to 24 micrograms per dose.

12. The method of claim 11, wherein the effective amount is from about 100 nanograms to about 1000 nanograms per dose.

13. The method of claim 1, wherein the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDASRLDLRIG (C/R)IVTAKKHPDADSLYVEEVDVGEAAPRTVVSGLVNHVPLEQMQNRMVVL LCNLKPAKMRGVLSQAMVMCASSPEKVEILAPPNGSVPGDRITFDAFPGEPDK ELNPKKKIWEQIQPDLHTNAECVATYKGAPFEVKGKGVCRAQTMANSGIK, wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues.

14. The method of claim 13, wherein the homology is at least about ninety-five percent.

15. The method of claim 1, wherein the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDVSRLDLRIG (C/R)IITARKHPDADSLYVEEVDVGEIAPRTVVSGLVNHVPLEQMQNRMVIL LCNLKPAKMRGVLSQAMVMCASSPEKIEILAPPNGSVPGDRITFDAFPGEPDK ELNPKKKIWEQIQPDLHTNDECVATYKGVPFEVKGKGVCRAQTMSNSGIK, wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues.

16. The method of claim 15, wherein the homology is at least about ninety-five percent.

17. The method of claim 1, wherein the agent is endothelial monocyte activating polypeptide II.

18. The method of claim 17, wherein the endothelial monocyte activating polypeptide II is murine endothelial monocyte activating polypeptide II.

19. The method of claim 17, wherein the endothelial monocyte activating polypeptide II is human endothelial monocyte activating polypeptide II.

20. The method of claim 17, wherein the endothelial monocyte activating polypeptide II is recombinant endothelial monocyte activating polypeptide II.

21. The method of claim 1, wherein the tumor is too small for intratumoral injection.

22. The method of claim 21, wherein the diameter of the tumor is less than or equal to about two millimeters.

23. A method of inhibiting the growth of endothelial cells, comprising contacting the endothelial cells with an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to inhibit growth of the endothelial cells.

24. The method of claim 23, wherein the endothelial cells are aortic endothelial cells.

25. A method of inhibiting the formation of blood vessels in a subject, comprising administering to the subject an effective amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, thereby inhibiting the formation of blood vessels in the subject.

26. The method of claim 25, wherein the subject is a mouse.

27. The method of claim 25, wherein the agent is administered subcutaneously, intravascularly, intraperitoneally, topically, or intramuscularly.

28. The method of claim 25, wherein the effective amount is from about 10 nanograms to about 24 micrograms.

29. The method of claim 28, wherein the effective amount is from about 100 nanograms to about 1 microgram.

30. The method of claim 25, wherein the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDASRLDLRIG (C/R)IVTAKKHPDADSLYVEEVDVGEAAPRTVVSGLVNHVPLEQMQNRMVVL LCNLKPAKMRGVLSQAMVMCASSPEKVEILAPPNGSVPGDRITFDAFPGEPDK ELNPKKKIWEQIQPDLHTNAECVATYKGAPFEVKGKGVCRAQTMANSGIK, wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues.

31. The method of claim 30, wherein the homology is about ninety-five percent.

32. The method of claim 25, wherein the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDVSRLDLRIG (C/R)IITARKHPDADSLYVEEVDVGEIAPRTVVSGLVNHVPLEQMQNRMVIL LCNLKPAKMRGVLSQAMVMCASSPEKIEILAPPNGSVPGDRITFDAFPGEPDK ELNPKKKIWEQIQPDLHTNDECVATYKGVPFEVKGKGVCRAQTMSNSGIK, wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues.

33. The method of claim 32, wherein the homology is at least about ninety-five percent.

34. The method of claim 25, wherein the agent is endothelial monocyte activating polypeptide II.

35. The method of claim 25, wherein the endothelial monocyte activating polypeptide II is murine endothelial monocyte activating polypeptide II.

36. The method of claim 25, wherein the endothelial monocyte activating polypeptide II is human endothelial monocyte activating polypeptide II.

37. The method of claim 25, wherein the endothelial monocyte activating polypeptide II is recombinant endothelial monocyte activating polypeptide II.

38. A method of treating a condition involving the presence of excess blood vessels in a subject, comprising the method of claim 25.

39. The method of claim 25, wherein the condition involves the presence of excess blood vessels in the eye.

40. The method of claim 39, wherein the condition is a retinopathy.

41. The method of claim 40, wherein the retinopathy is diabetic retinopathy.

42. The method of claim 40, wherein the retinopathy is sickle cell retinopathy.

43. The method of claim 40, wherein the retinopathy is retinopathy of prematurity.

44. The method of claim 40, wherein the retinopathy is age related macular degeneration.

45. A method of treating a tumor in a subject, comprising

administering to the subject an amount of an agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide, effective to treat the tumor, wherein the endothelial monocyte activating polypeptide II is administered subcutaneously or intraperitoneally; and intravenously, intracranially, or intramorally.

46. The method of claim 45, wherein the tumor is a glioblastoma.

47. The method of claim 45, wherein the agent is administered intratumorally by positive pressure microinfusion.

48. The method of claim 45, wherein the endothelial monocyte activating polypeptide II-derived polypeptide is at least about ninety percent homologous to the sequence (S/M/G)KPIDASRLDLRIG (C/R)IVTAKKHPDADSLYVEEVDVGEAAPRTVVSGLVNHVPLEQMQNRMVVL LCNLKPAKMRGVLSQAMVMCASSPEKVEILAPPNGSVPGDRITFDAFPGEPDK ELNPKKKIWEQIQPDLHTNAECVATYKGAPFEVKGKGVCRAQTMANSGIK, wherein the sequence is truncated by from zero to about three amino-terminal residues and from zero to about one hundred thirty-six carboxy-terminal residues.

49. A method for evaluating the ability of an agent to inhibit growth of endothelial cells, which comprises:

(a) contacting the endothelial cells with an amount of the agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide;

(b) determining the growth of the endothelial cells, and

(c) comparing the amount of growth of the endothelial cells determined in step (b) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit growth of endothelial cells.

50. A method for evaluating the ability of an agent to inhibit the formation of a blood vessel in a cellular environment, which comprises:

(a) contacting the cellular environment with an amount of the agent, selected from endothelial monocyte activating polypeptide II and an endothelial monocyte activating polypeptide II-derived polypeptide;

(b) determining whether or not a blood vessel has formed in the cellular environment, and

(c) comparing the amount of blood vessel growth determined in step (b) with the amount determined in the absence of the agent in the cellular environment, thus evaluating the ability of the agent to inhibit blood vessel formation in the cellular environment.

51. A pharmaceutical composition which comprises an agent capable of inhibiting blood vessel formation and a pharmaceutically acceptable carrier.

52. The pharmaceutical composition of claim 51, wherein the carrier is a diluent, an aerosol, a topical carrier, an aquous solution, a nonaqueous solution or a solid carrier.

FIG. 1

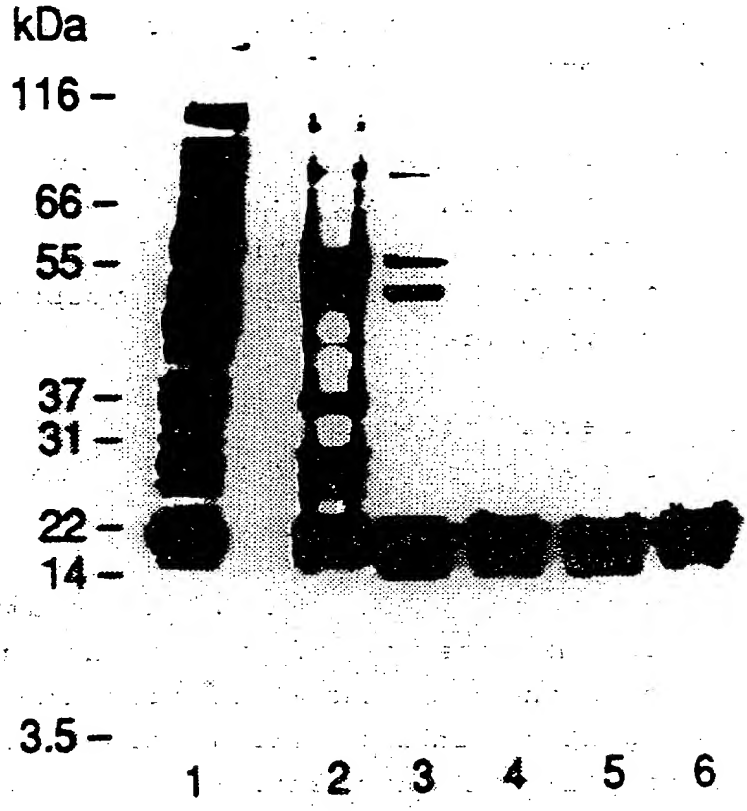


FIGURE 2A

FIGURE 2B

FIGURE 2C

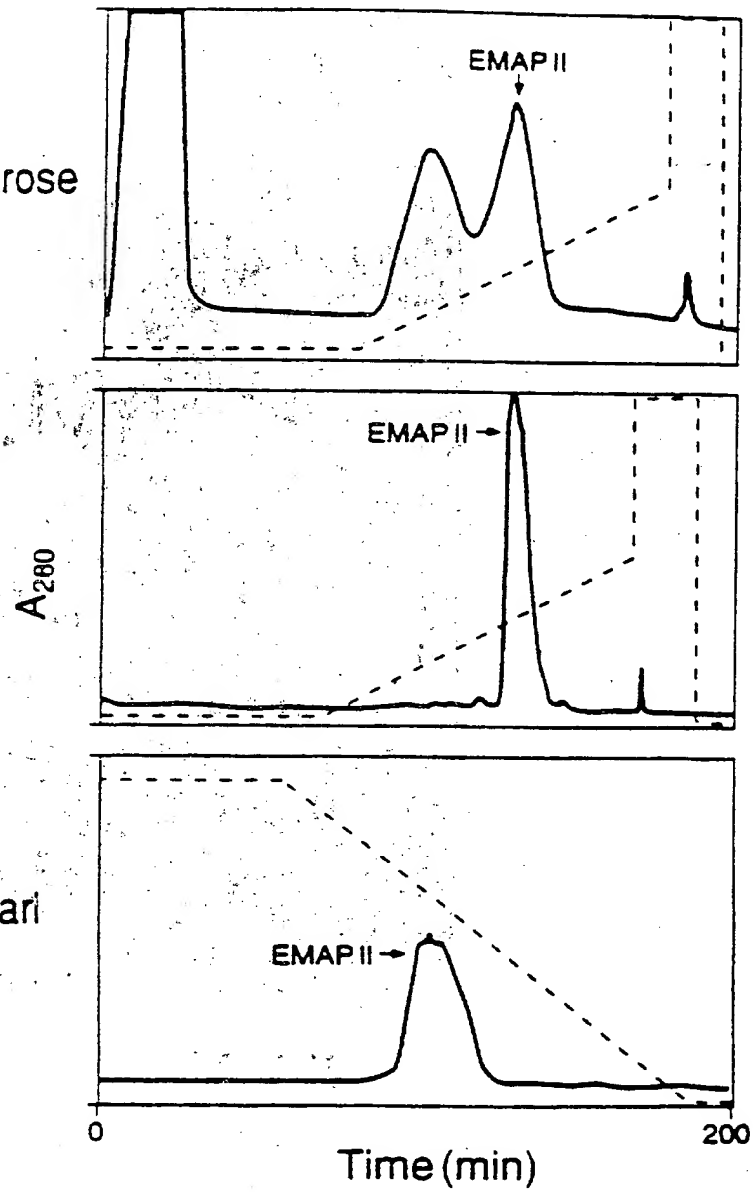


FIG. 3A FIG. 3B

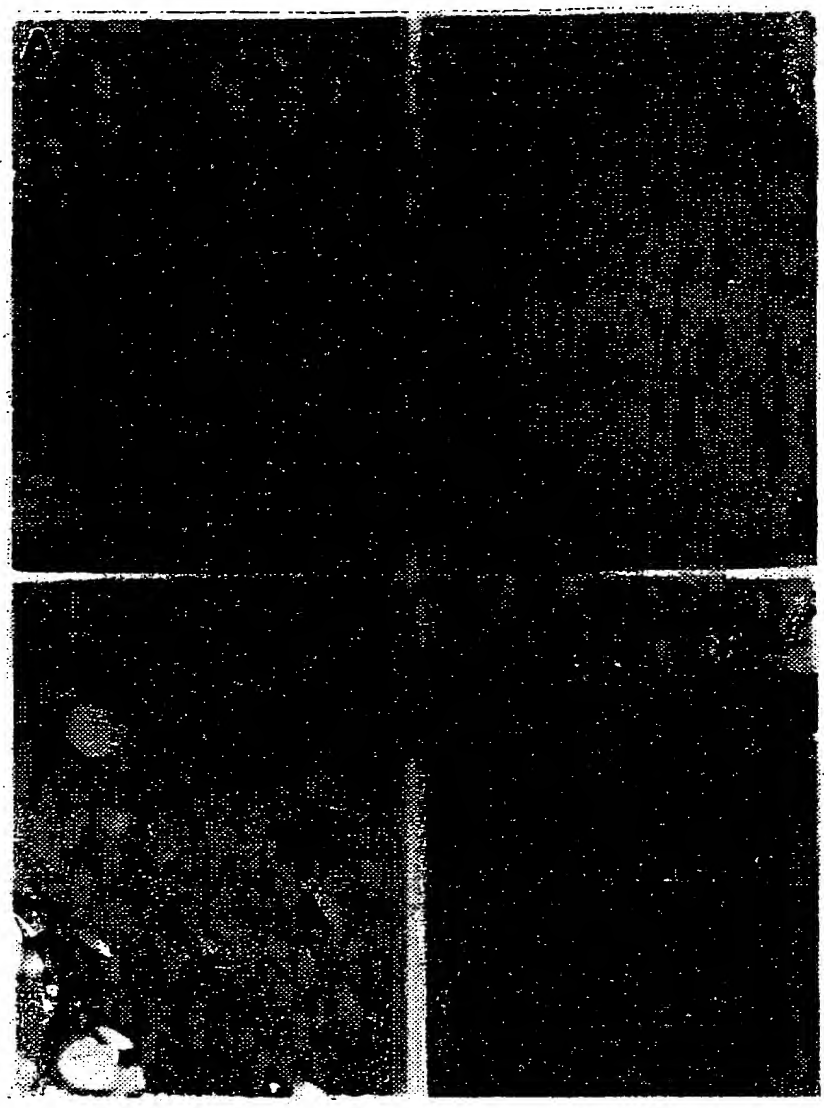


FIG. 3C FIG. 3D

FIG. 3E

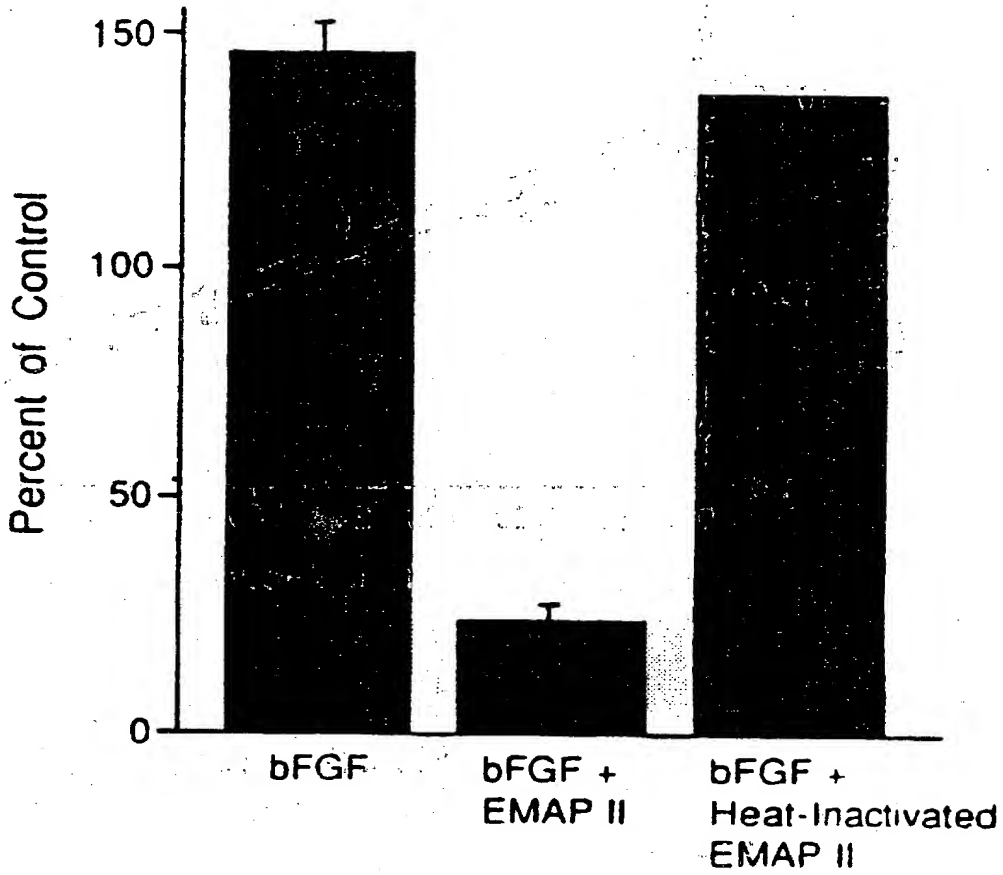


FIGURE 4A

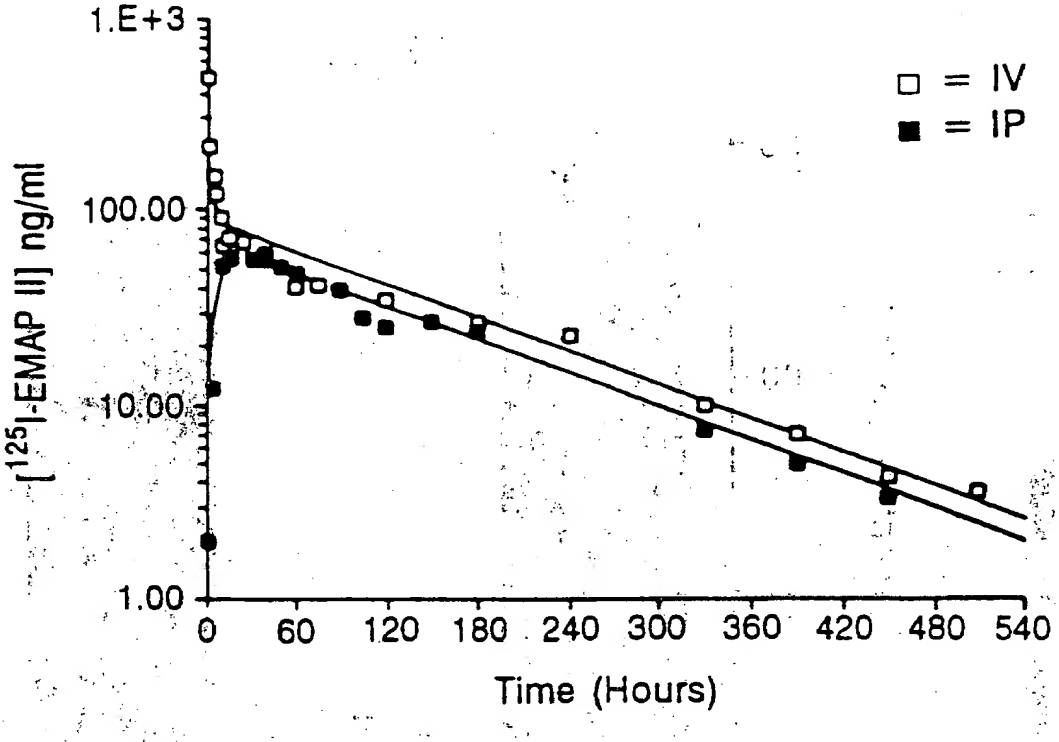


FIGURE 4B

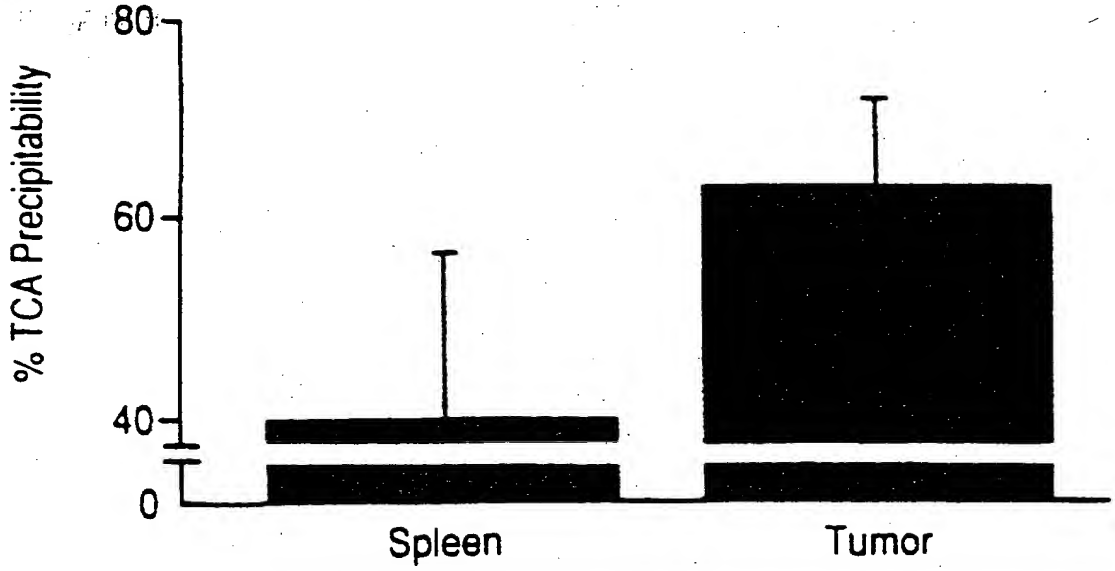


FIGURE 4C

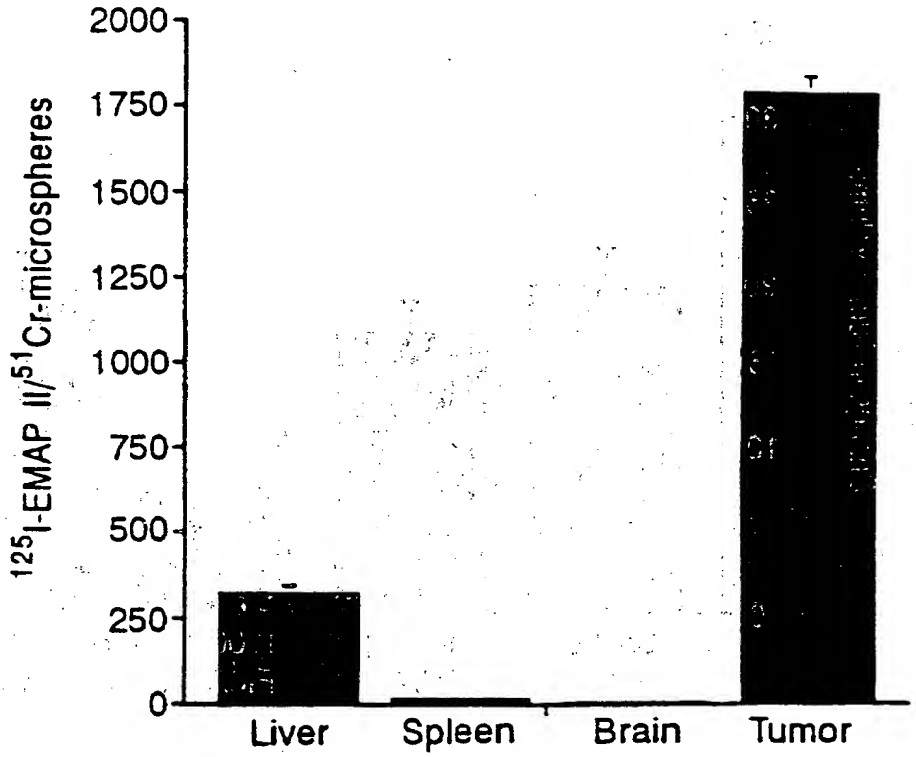
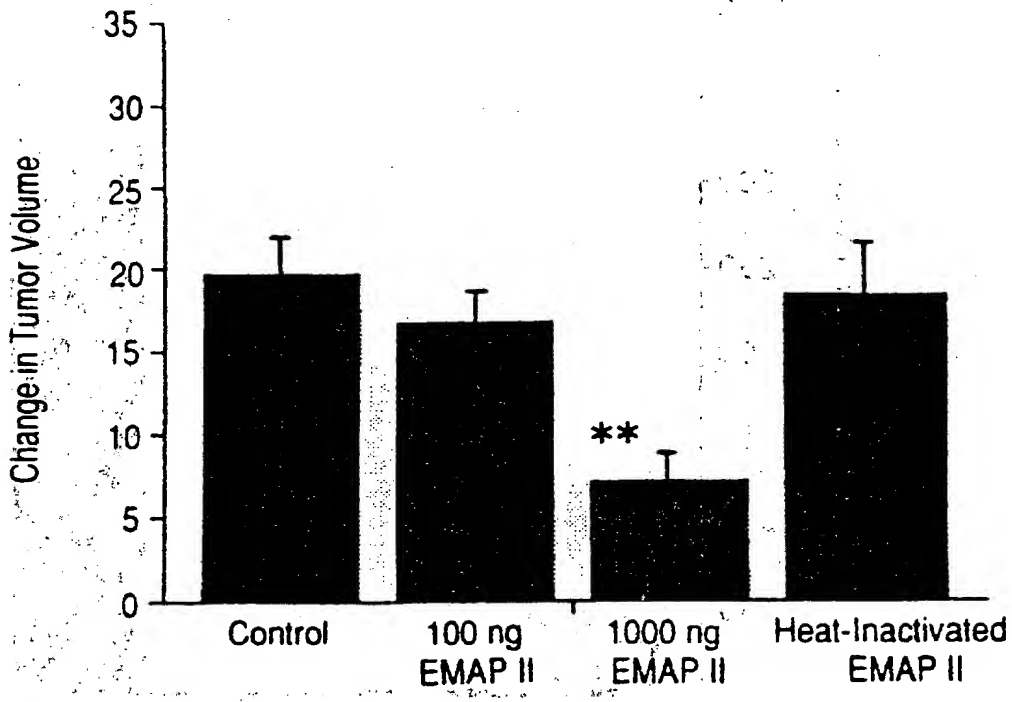


FIG. 5A



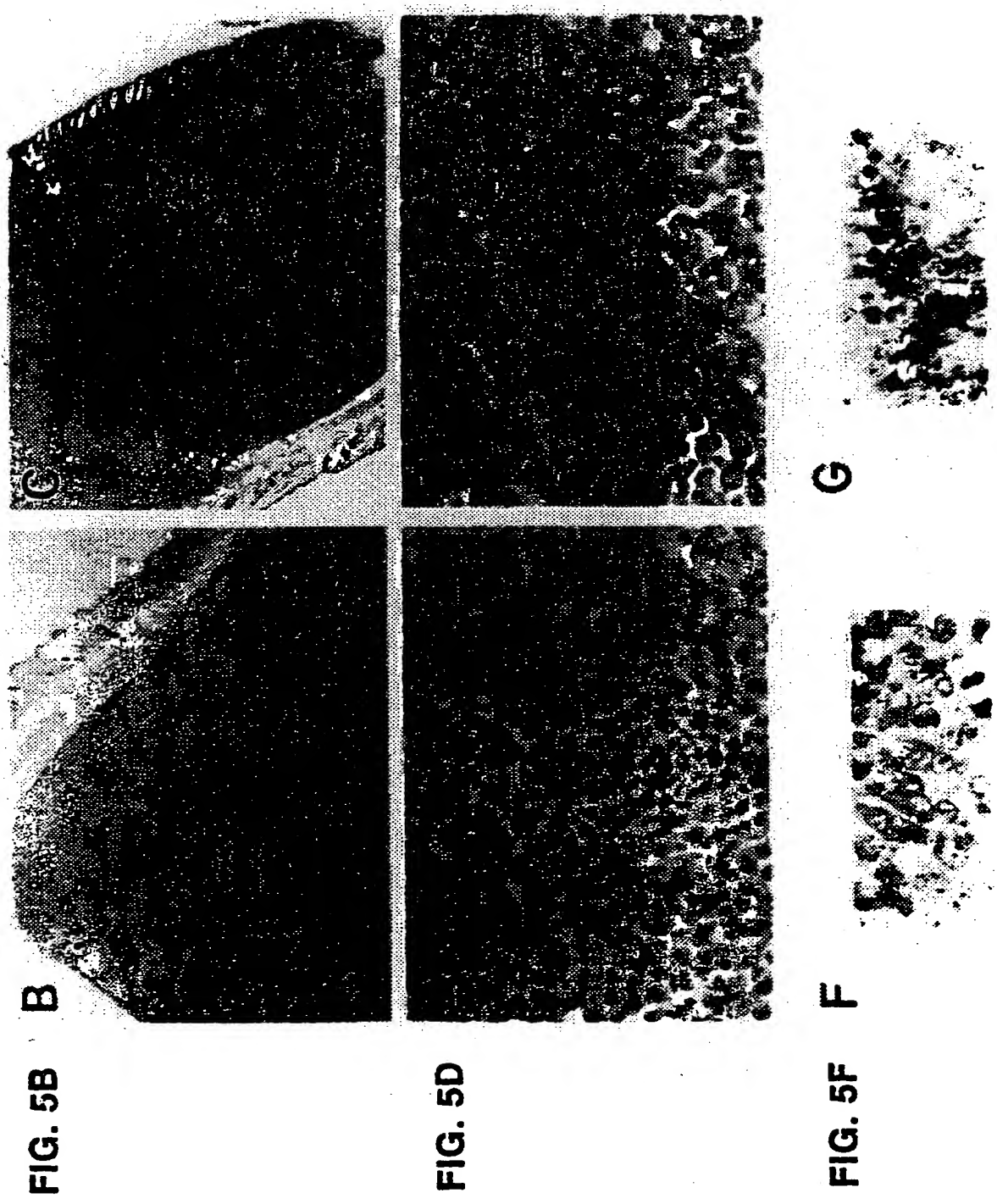


FIG. 5C FIG. 5E FIG. 5G

FIG. 5B FIG. 5D FIG. 5F

FIG. 6A

FIG. 6B

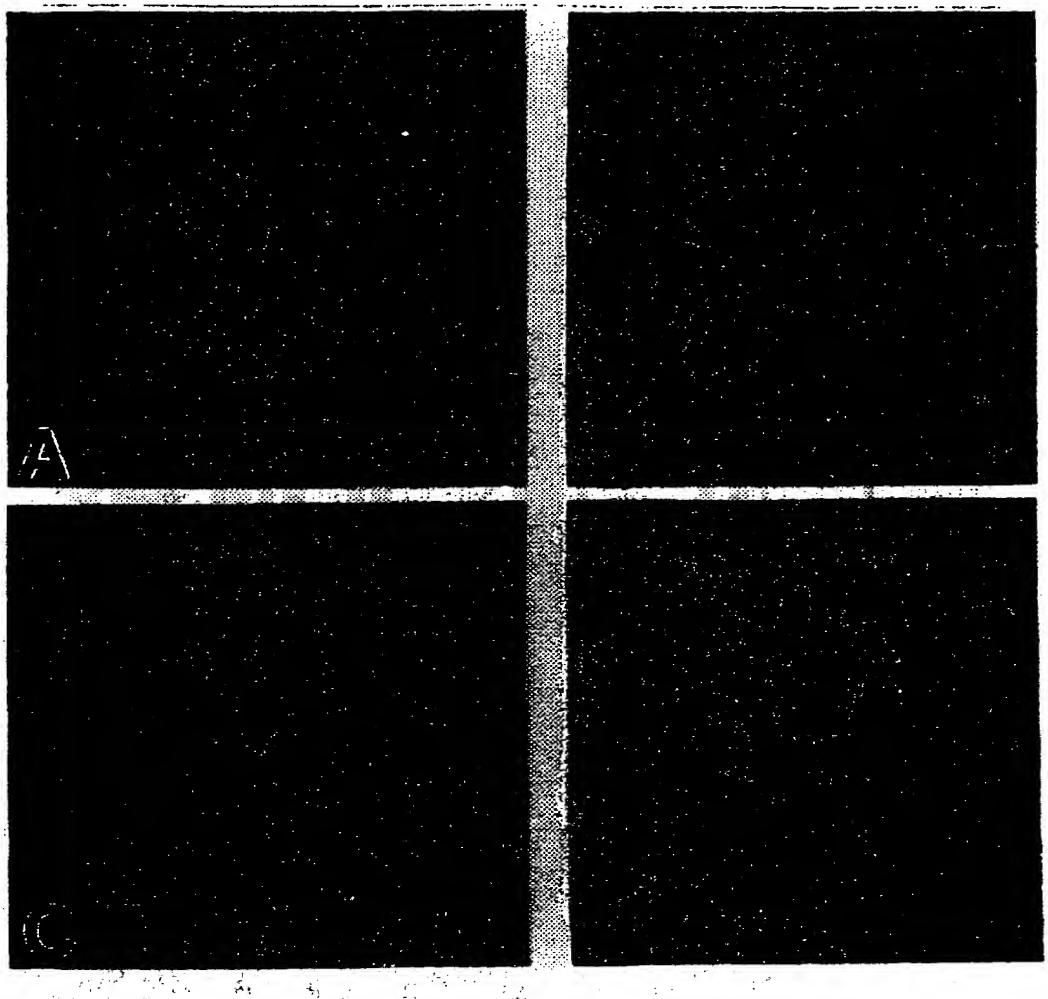


FIG. 6C

FIG. 6D

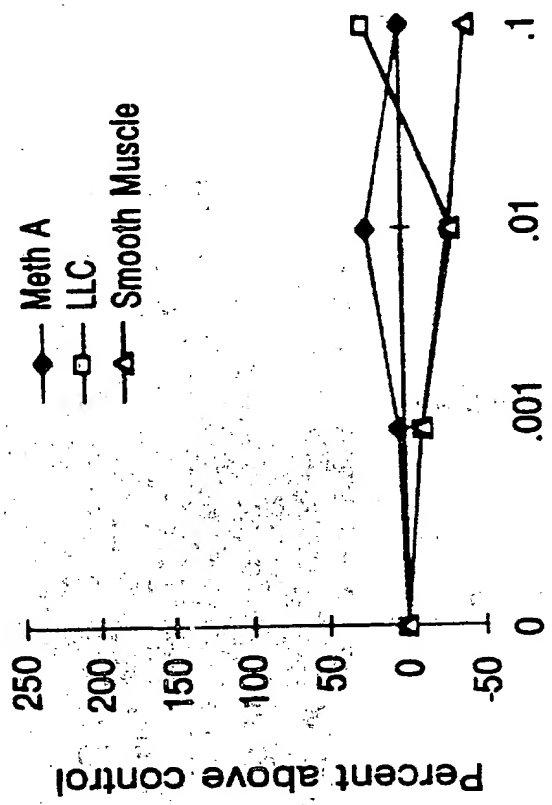


FIG. 6F

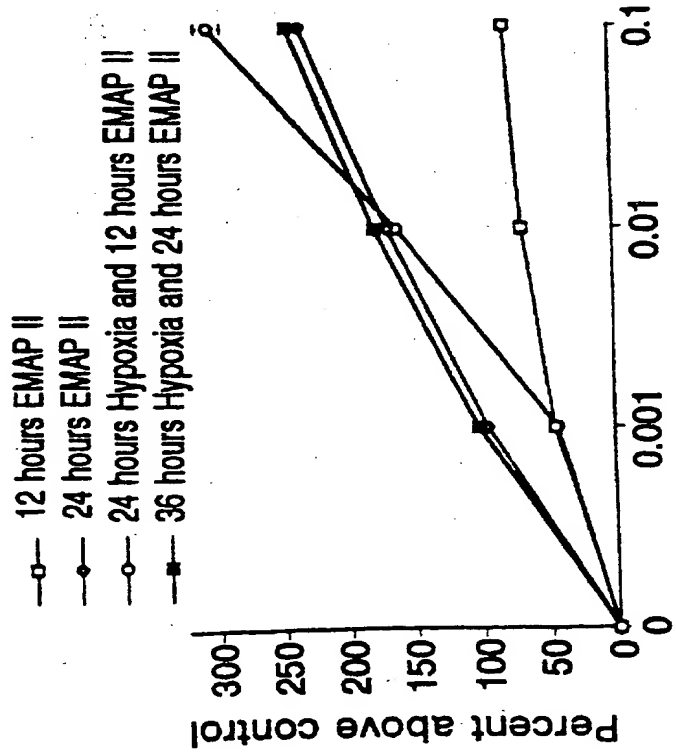


FIG. 6E

FIG. 7

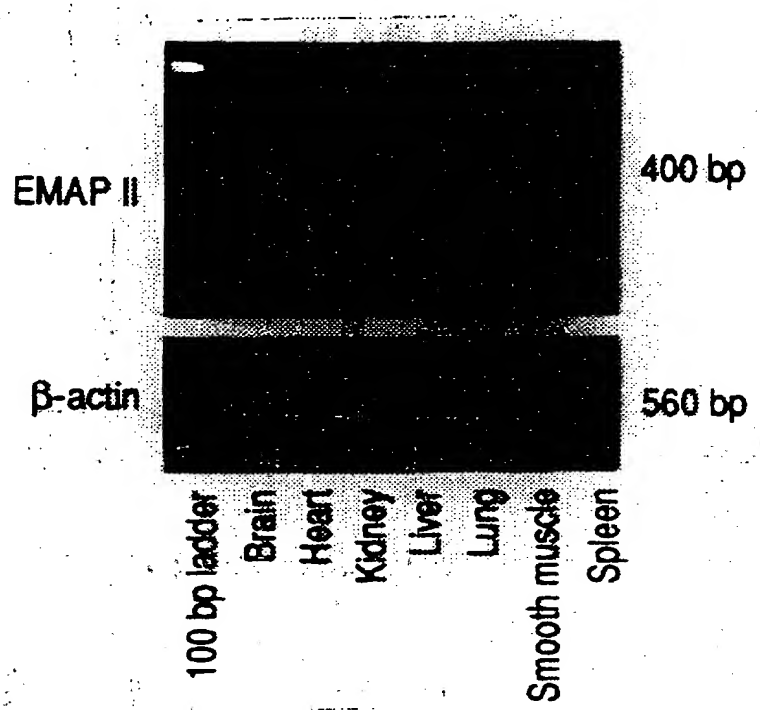


FIG. 8A

FIG. 8B

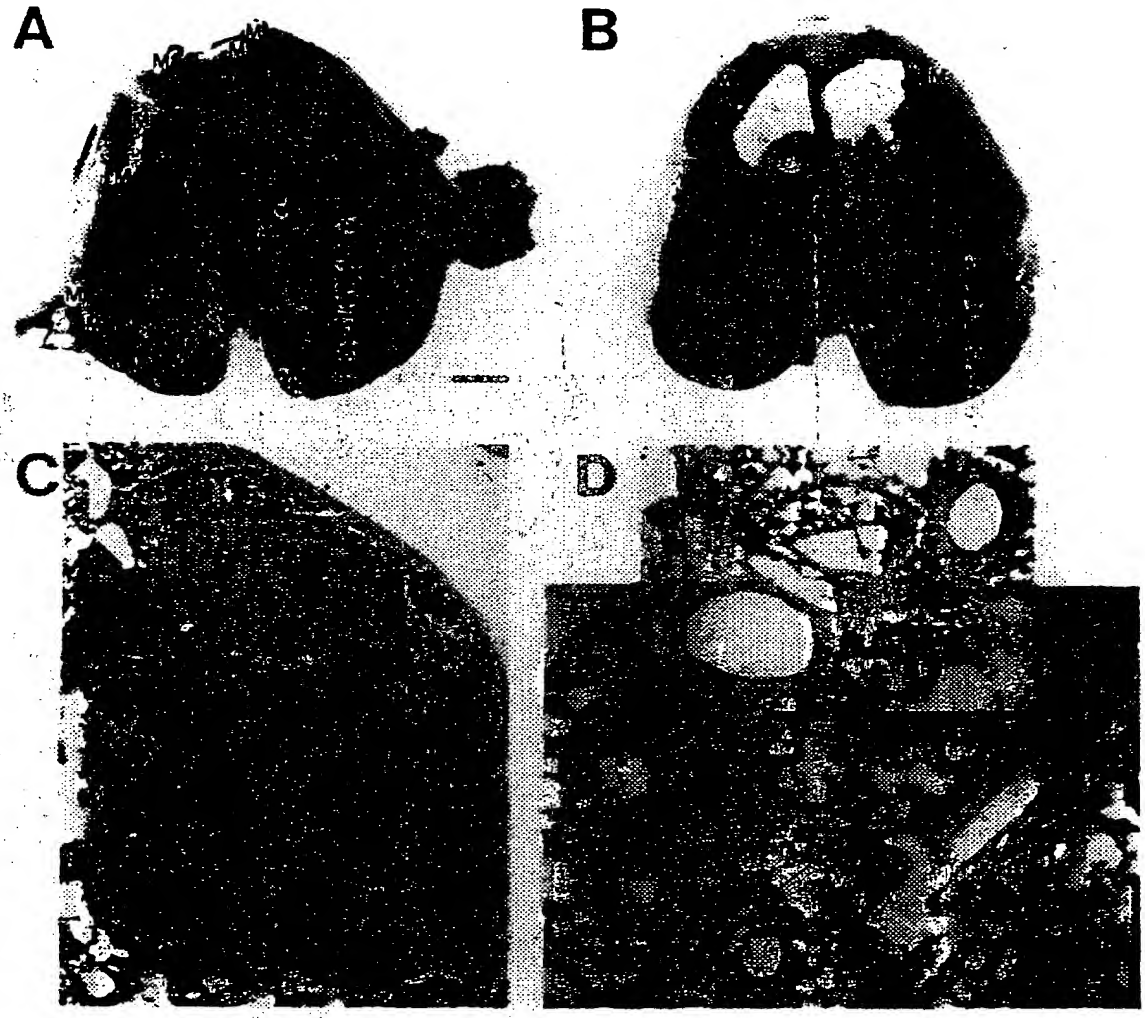


FIG. 8C

FIG. 8D

FIG. 8E-1

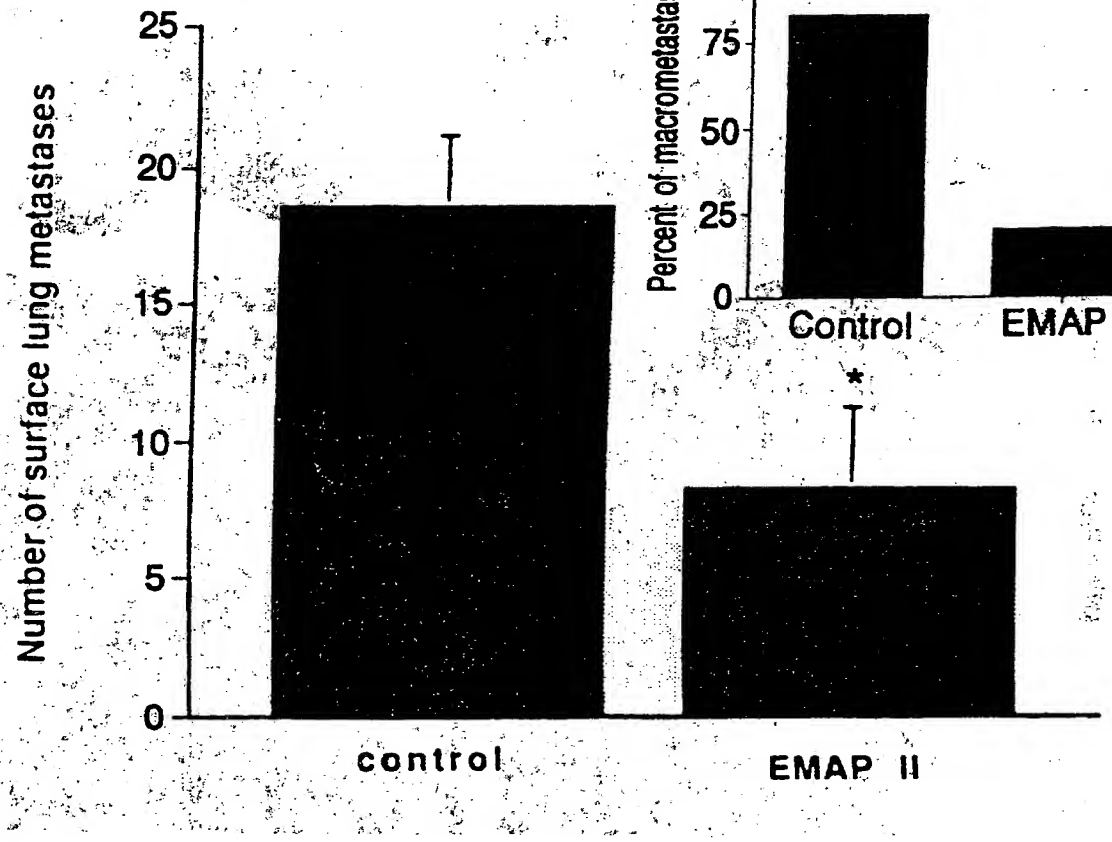
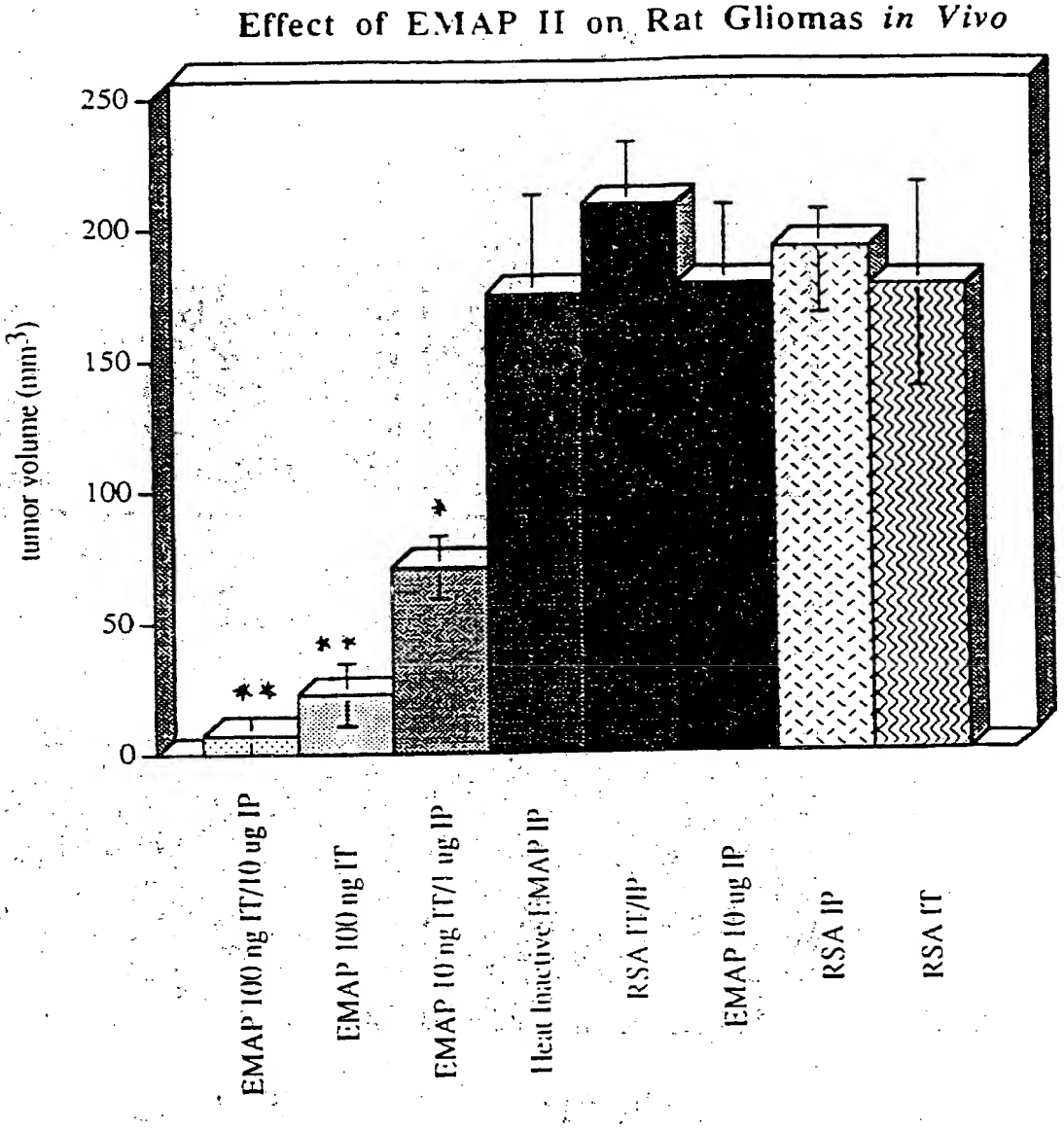


FIG. 8E-2

FIG. 9A



(IT=intratumoral;IP=intraperitoneal;RSA=rat serum albumin)

FIG. 9B

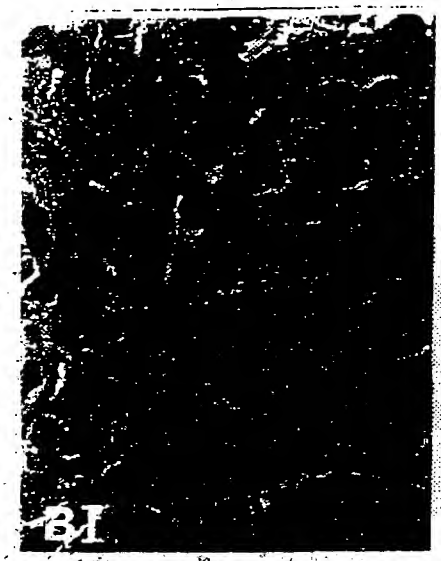


FIG. 9C

FIG. 9D

FIG. 9E

FIG. 9F

EMAP II Inhibits Growth of C 6 Gliomas in Immunocompromised Mice.

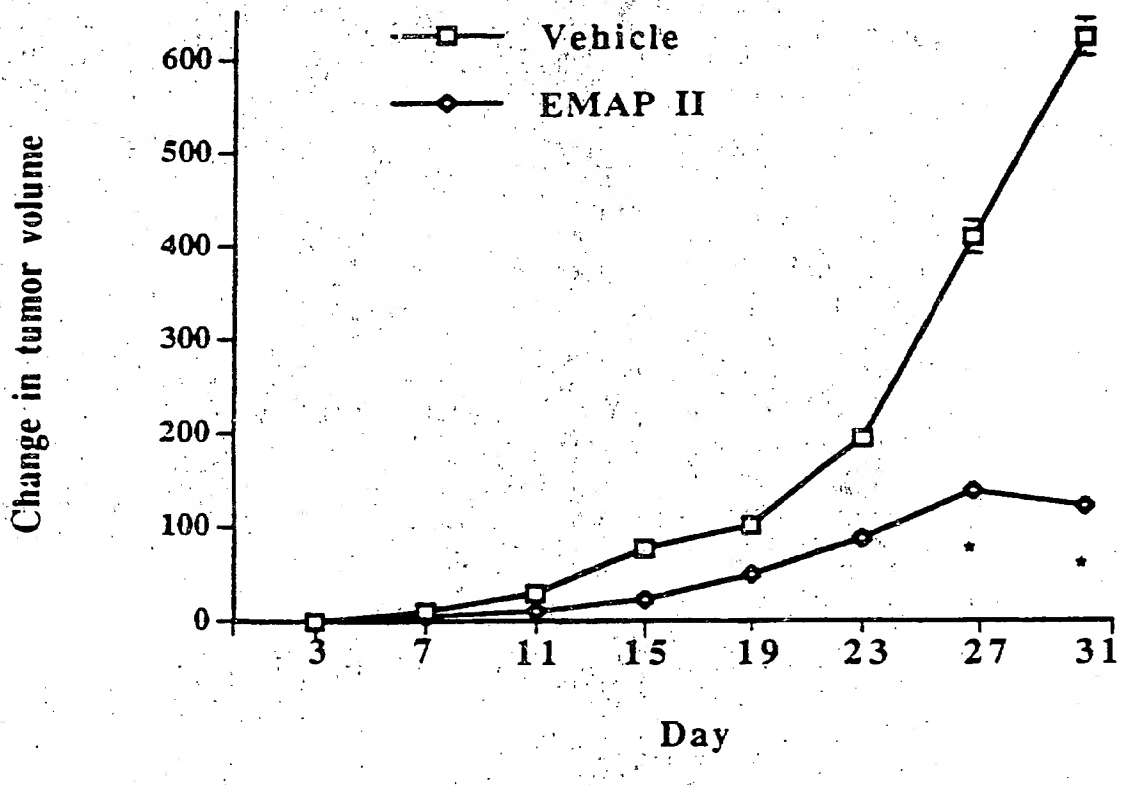


FIG. 10B

FIG. 10A

FIG. 10C

FIG. 10D

FIG. 10E

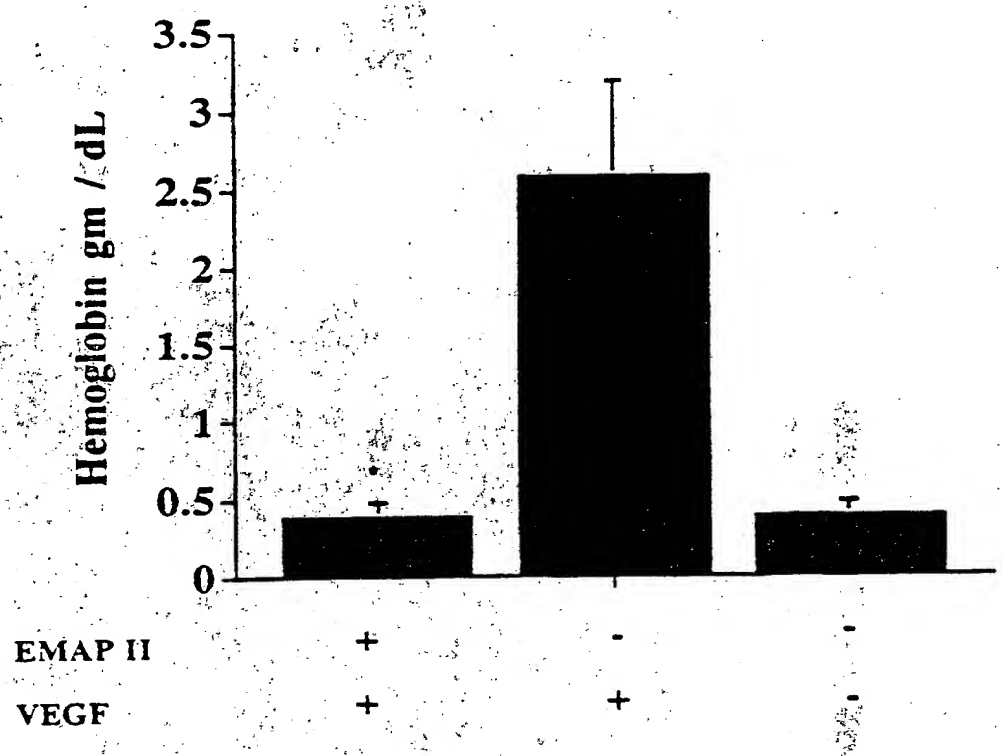
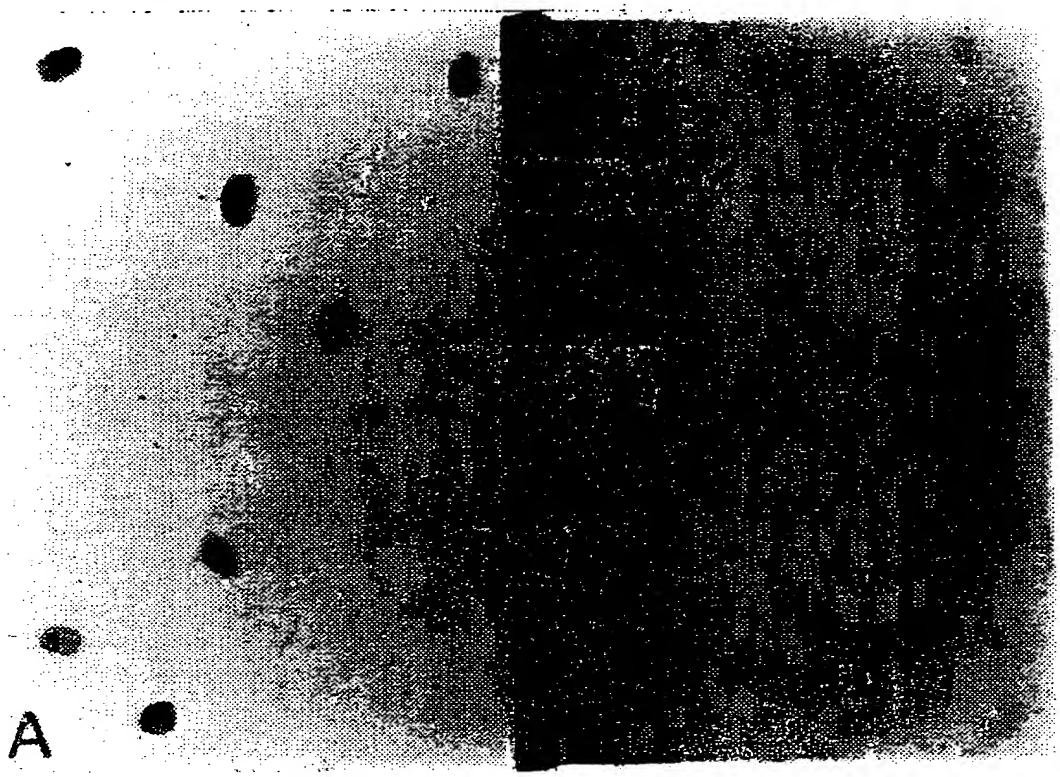


FIG. 11A FIG. 11B



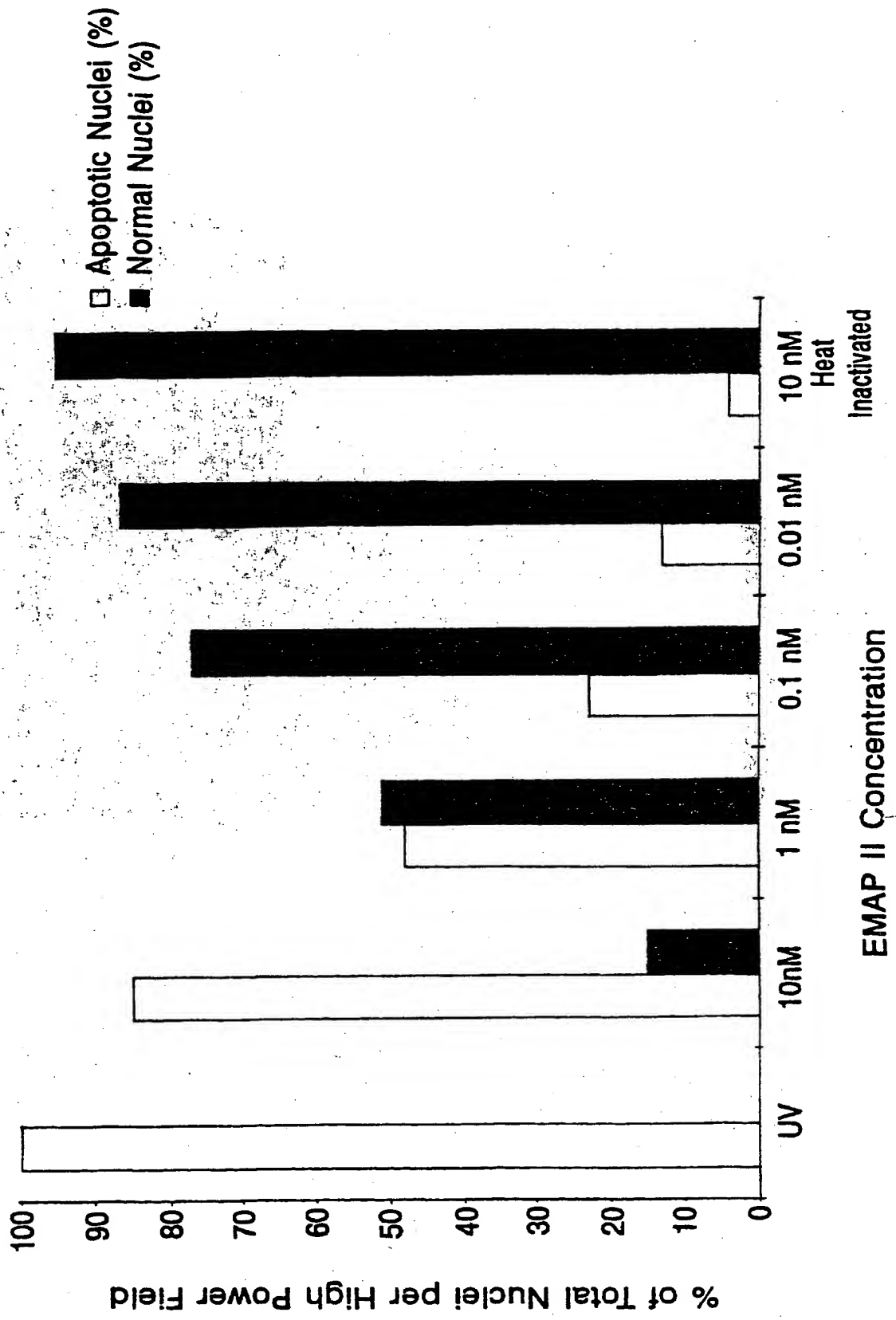
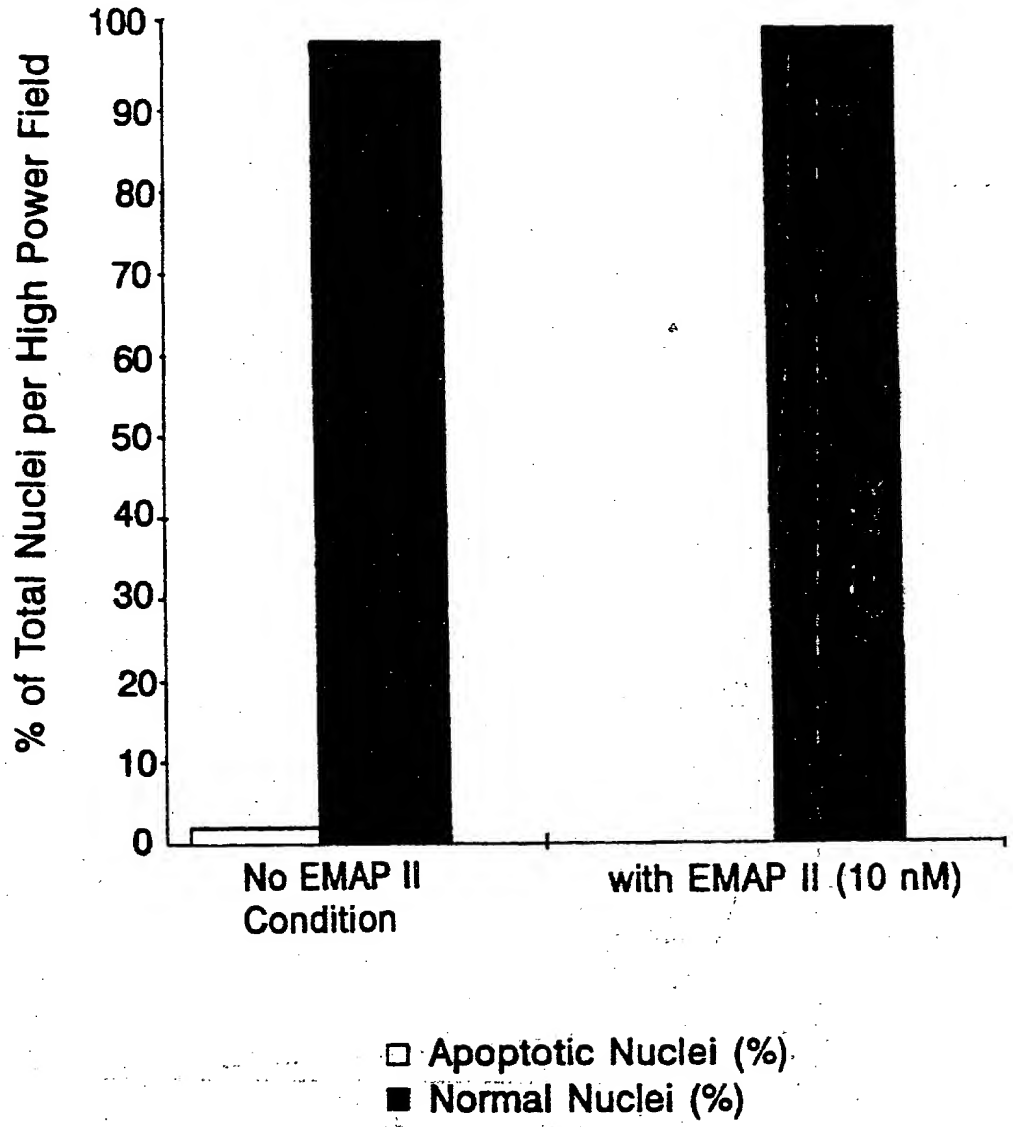


FIG. 11C

FIG. 11D



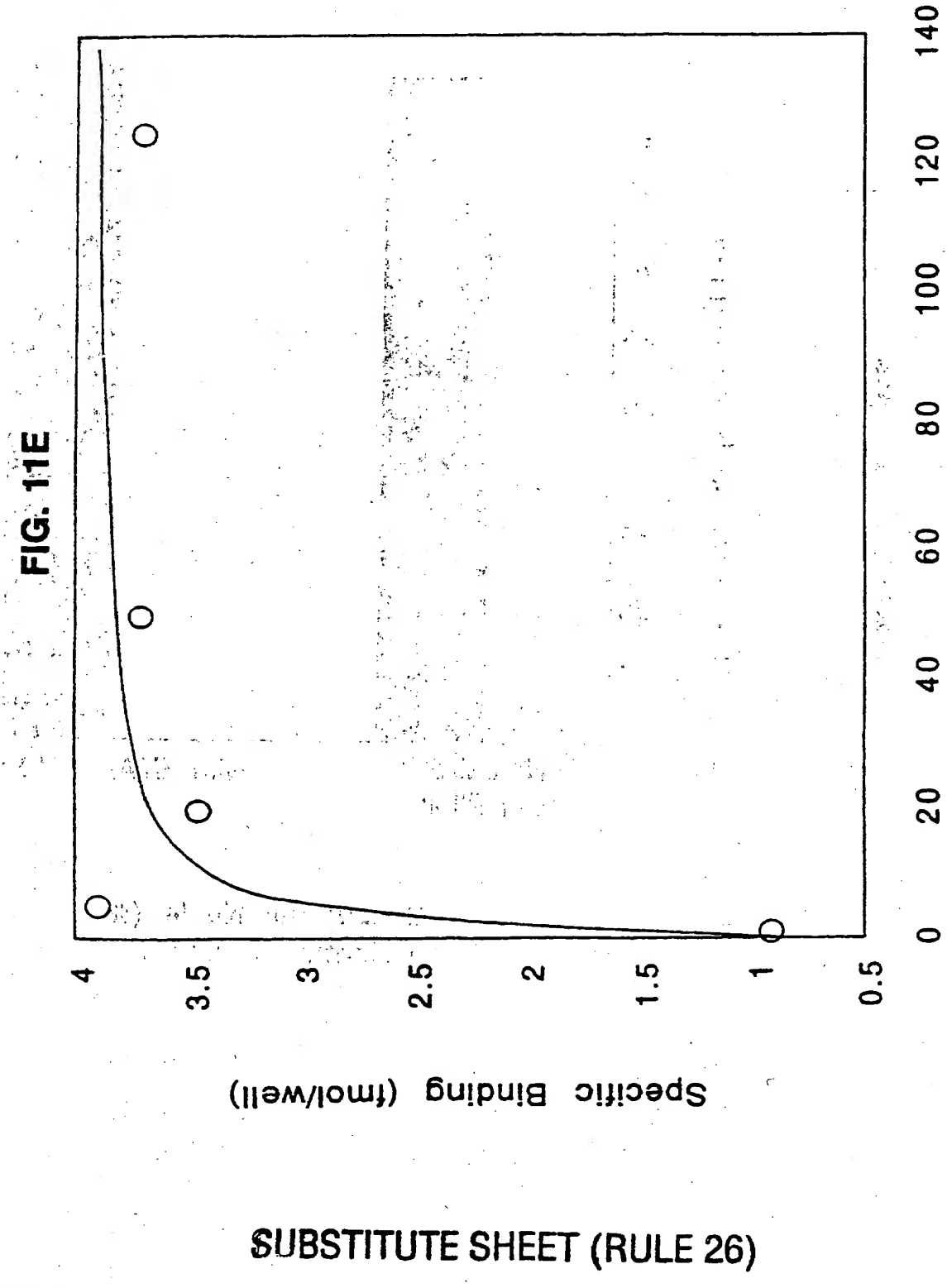


FIG. 11E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/15007

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/17; C12Q 1/02
US CL :435/29; 514/12, 21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/29; 514/2, 12, 21; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG
search terms: emap, endothelial monocyte activating, angiogenesis, neovascularization, retinopathy, endothelial

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KAO et al. Endothelial Monocyte-activating Polypeptide II. The Journal Of Biological Chemistry. 05 October 1992, Volume 267, Number 28, pages 20239-20247.	1-52
X	KAO et al. Characterization of a Novel Tumor-derived Cytokine. The Journal Of Biological Chemistry. 07 October 1994, Volume 269, Number 40, pages 25106-25119, especially page 25108, column 2, last paragraph, page 25109.	23-37, 51, 52
X	US 5,382,514 A (PASSANITI et al.) 17 January 1995, see entire document, especially column 3, lines 50-64, column 11, lines 5-18.	49-52

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 NOVEMBER 1996	29 NOV 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JW for JEFFREY E. RUSSEL
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/15007

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,019,556 A (SHAPIRO et al.) 28 May 1991, see entire document, especially column 8, line 40 through column 10, line 16.	1-12, 21-29, 38, 45-47, 51, 52
X	US 5,198,423 A (TAGUCHI et al.) 30 March 1993, see entire document, especially column 14, lines 30-50.	1-12, 21-29, 38, 45-47, 50-52
X	US 5,202,116 A (BROWN et al.) 13 April 1993, see entire document, especially column 19, lines 20-33.	49
X	US 5,284,827 A (MAIONE et al.) 08 February 1994, see entire document, especially column 4, lines 19-20, column 9, line 50 through column 10, line 37, column 13, line 38 through column 14, line 63.	1-12, 21-29, 38-47, 49-52
X,P	SCHWARZ et al. Endothelial-Monocyte Activating Polypeptide (EMAP) II, a Novel Antiangiogenic Protein, Suppresses Tumor Growth and Induces Apoptosis in Endothelial Cells. American Heart Association Supplement to Circulation. 15 October 1995, Volume 92, Number 8, Supplement, pages I-7 - I-8, abstract no. 0034, see entire abstract.	1-38, 45-52
X	WO 95/09180 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 06 April 1995, see entire document, especially page 6, lines 22-34, page 29, line 4 through page 31, line 9; page 79, lines 10-21, page 113, lines 24-29, claim 68.	1-38, 45-48, 51, 52

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*